UNIVERSITY OF ADELAIDE
MAY
5
1958

THE AUSTRALIAN JOURNAL OF EXPERIMENTAL BIOLOGY AND MEDICAL SCIENCE

EDITOR-IN-CHIEF
MARK L. MITCHELL

CO-EDITOR
G. M. BADGER

VOL. XXXV

PUBLISHED BY THE UNIVERSITY OF ADELAIDE

PRINTED AT THE GRIFFIN PRESS, MARLBOROUGH PLACE, ADELAIDE
1957

B570.5
Au78

CONTENTS

PART 1. FEBRUARY, 1957.

PAGE

NANCY ATKINSON: Lysogenicity and lysis patterns in the Salmonellas. 11. The bacteriophage grouping scheme compared with other schemes using bacteriophage for exact strain identification in the Salmonellas - 1

L. B. COBBIN AND R. H. THORP: Some pharmacological properties of himandrine, an alkaloid derived from *Himantandra baccata* - - - - 15

P. S. VENKATACHALAM AND I. KALDOR: Studies on intermediary iron metabolism. IX. The effects of partial hepatectomy in rats - - - 25

ALLAN J. DAY, COLIN J. SCHWARTZ, GWENDOLINE K. WILKINSON AND J. A. PETERS: The effect of uranium acetate on serum lipids and on atherosclerosis in cholesterol fed rabbits - - - - - - - - - 31

N. T. HINKS AND A. BOLLIGER: Glucuronuria in a herbivorous marsupial *Trichosurus vulpecula* - - - - - - - - - - - 37

BRYAN HUDSON AND G. A. BENTLEY: The biological assay of melanocyte stimulating hormone - - - - - - - - - - 45

PATRICIA E. LIND AND F. M. BURNET: Recombination between virulent and non-virulent strains of influenza virus. 1. The significance of heterozygosis - - - - - - - - - - - - - - 57

PATRICIA E. LIND AND F. M. BURNET: Recombination between virulent and non-virulent strains of influenza virus. 2. The behaviour of virulence markers on recombination - - - - - - - - - 67

M. J. RAND: The effect of potassium and acetylcholine on the response of the guinea-pig jejunum to histamine - - - - - - - 79

B. R. A. O'BRIEN: Evidence in support of an axial metabolic gradient in the earthworm - - - - - - - - - - - - - 83

U of M Bindery SEP 1958

PART 2. APRIL, 1957.

PAGE

HENRY T. YOST, JR., AND ROBERT A. DARROW: Failure to inactivate urease with near-infrared radiation - - - - - - - - - - 93

MARY C. DAWBARN, DENISE C. HINE AND JEAN SMITH: The determination of vitamin B_{12}-activity in the organs and excreta of sheep. 4. The separation of vitamin B_{12}-active factors from rumen contents by paper ionophoresis - - - - - - - - - - - - - 97

I. G. JARRETT AND B. J. POTTER: The metabolism of lower fatty acids in diabetic sheep - - - - - - - - - - - - - - 103

J. F. A. P. MILLER AND P. M. DE BURGH: Ectromelia virus multiplication in regenerating mouse liver - - - - - - - - - - - 115

I. KALDOR AND MARJORIE POWELL: Studies on intermediary iron metabolism. X. The influence of age and sex on the storage of supplemental dietary iron in the rat - - - - - - - - - - 123

WINIFRED G. NAYLER: Cardiac metabolism. IV. Seasonal variation. Seasonal variation in metabolism and drug sensitivity of the isolated toad heart (*Bufo marinus*) - - - - - - - - - - 131

R. G. GIOVANELLI: The application of diffuse reflection spectrophotometry to chemical analysis - - - - - - - - - - - - 143

G. A. BENTLEY AND BRYAN HUDSON: A comparison of methods for the isolation of melanocyte stimulating hormone from blood - - - - - 157

GEORGE READ: A metabolic aspect of renal hypertension - - - - - 163

P. E. HUGHES: Studies on the intracellular distribution of protein-bound amino-azo dyes in rat liver - - - - - - - - - - 173

J. J. LAWRENCE: The invasion of the chick embryo by ectromelia virus after inoculation on the chorioallantoic membrane - - - - - - 179

A. J. BEARUP: Experimental vectors of the first larval stage of *Dibothriocephalus latus* (cestoda) in Australia - - - - - - - - 187

PART 3. JUNE, 1957.

PAGE

NANCY ATKINSON AND L. R. BULLAS: Salmonella bacteriophages. 6. Some heat resistant phages - - - - - - - - - - - - 193

P. FANTL AND H. A. WARD: Comparison of blood clotting in marsupials and man - - - - - - - - - - - - - - - 209

F. M. BURNET AND PATRICIA E. LIND: Reversion to virulence in an influenza virus mutant - - - - - - - - - - - - - 225

WINIFRED G. NAYLER: Cardiac metabolism : ionic changes. Influence of calcium ions, 9α-fluorohydrocortisone and cardiac glycosides on the isolated toad heart - - - - - - - - - - - - 241

L. B. COBBIN AND R. H. THORP: The spasmolytic actions of some synthetic compounds related to papaverine - - - - - - - - - 249

D. S. KRONFELD: The effects on blood sugar and ketone bodies of butyrate, acetate and β-OH butyrate infused into sheep - - - - - - 257

DENISE C. HINE, MARY C. DAWBARN AND JEAN SMITH: The effect of copper on the response to vitamin B_{12} and methionine of a mutant strain of *Escherichia coli*: a modification of the basal medium - - - - 267

MARY C. DAWBARN, DENISE C. HINE AND JEAN SMITH: The determination of vitamin B_{12}-activity in the organs and excreta of sheep. 5. The effect of cobalt deficiency on the vitamin B_{12} content of the blood plasma - - - - - - - - - - - - - - 273

ALLAN J. DAY, GWENDOLINE K. WILKINSON, COLIN J. SCHWARTZ AND J. A. PETERS: Changes in serum lipids and in aortic atherosclerosis following toluidine blue and heparin administration to cholesterol fed rabbits 277

A. SHULMAN: Some aspects of the pharmacology of 5-phenyl-thiazolidine-2 : 4-dione (2:4-dihydroxy-5-phenylthiazole) - - - - - - 289

PART 4. AUGUST, 1957.

PAGE

J. R. GODING AND D. A. DENTON: The effects of adrenal insufficiency and overdosage with DOCA on bilaterally adrenalectomized sheep - 301

MARY C. DAWBARN, DENISE C. HINE AND JEAN SMITH: The determination of vitamin B_{12}-activity in the organs and excreta of sheep. 6. Vitamin B_{12}-activity in portal blood - - - - - - - - - - 321

PETER WARNER: The detection of Murray Valley encephalitis antibodies in hens' eggs - - - - - - - - - - - - - 327

JOHN G. ATHERTON AND WILMA ANDERSON: Propagation in tissue culture of the virus of infectious laryngotracheitis of fowls - - - - - 335

F. R. MAGAREY AND W. E. STEHBENS: The blood pressure of sheep - - 347

P. S. VENKATACHALAM, ILSE BRADING, I. KALDOR AND E. P. GEORGE: Studies on intermediary iron metabolism. XI. The source of iron in the regenerated liver of the rat after hepatectomy - - - - - - 353

N. D. CROSBY AND G. WOOLLEY: Observations, using new methods, of the effects of neck compression on water-diuresis in rats - - - - 363

B. R. A. O'BRIEN: Tissue metabolism during posterior regeneration in the earthworm - - - - - - - - - - - - - 373

P. A. ROBERTSON AND W. V. MACFARLANE: Pain-producing substances from the stinging bush *Laportea moroides* - - - - - - - - 381

PART 5. OCTOBER, 1957.

PAGE

J. W. H. LUGG AND J. M. BOWNESS: A study of the relationships between the twenty-four hourly urinary outputs of 17-ketosteroids and creatinine, and the weights of twenty adult male subjects from each of six ethnic groups - - - - - - - - - - - - 395

A. SHULMAN AND GLENDA M. LAYCOCK: Further aspects of the analeptic activity of bemegride - - - - - - - - - - - 421

A. M. SNOSWELL: Flavokinase of *Lactobacillus arabinosus* 17.5 - - - 427

R. K. F. PAK POY: Electron microscopy of the marsupial renal glomerulus 437

COLIN J. SCHWARTZ, ALLAN J. DAY, J. ANDREW PETERS AND J. R. CASLEY-SMITH: Serum cholesterol and phospholipid levels of Australian aborigines - - - - - - - - - - - - - - 449

ALLAN J. DAY, COLIN J. SCHWARTZ, AND J. ANDREW PETERS: Serum lipid responses to heparin and to protamine in rabbits - - - - - 457

J. H. POPE, E. H. DERRICK AND I. COOK: *Toxoplasma* in Queensland. I. Observations on a strain of *Toxoplasma gondii* isolated from a bandicoot, *Thylacis obesulus* - - - - - - - - - - - 467

J. H. POPE, V. A. BICKS AND I. COOK: *Toxoplasma* in Queensland. II. Natural infections in bandicoots and rats - - - - - - - 481

WINIFRED G. NAYLER: The action of salicylate, dinitrophenol and cardiac glycosides on the isolated toad heart - - - - - - - - 491

H. K. WARD: The persistence of antibodies in the absence of antigenic stimulus - - - - - - - - - - - - - - - 499

PART 6. DECEMBER, 1957.

PAGE

F. M. BURNET AND PATRICIA E. LIND: The use of chilling in the analysis of the first stages of infection by influenza virus - - - - - - 507

F. M. BURNET, PATRICIA E. LIND AND BEVERLEY PERRY: The action of ribonuclease on the multiplication of influenza viruses in the deembryonated egg - - - - - - - - - - - - 517

PATRICIA E. LIND AND F. M. BURNET: Further studies of recombination between heat-inactivated virus and active virus - - - - - - 531

E. R. TRETHEWIE: The effect of butazolidine on the anaphylactic lung - 541

G. J. V. NOSSAL: The immunological response of foetal mice to influenza virus - - - - - - - - - - - - - - - 549

A. SHULMAN AND GLENDA M. LAYCOCK: The analeptic activity of bemegride to structurally unrelated hypnotics - - - - - - - 559

J. W. LEGGE, A. S. MORIESON, G. E. ROGERS AND M. A. MARGINSON: The chromatography and countercurrent distribution of secretin - - 569

R. K. F. PAK POY: Electron microscopy of the amphibian renal glomerulus 583

C. A. MIMS: Rift Valley fever virus in mice. VI. Histological changes in the liver in relation to virus multiplication - - - - - - 595

S. ROSE AND J. F. NELSON: The direct effect of oestradiol on the pars distalis 605

THE GRIFFIN PRESS

LYSOGENICITY AND LYSIS PATTERNS IN THE SALMONELLAS

11. THE BACTERIOPHAGE GROUPING SCHEME COMPARED WITH OTHER SCHEMES USING BACTERIOPHAGE FOR EXACT STRAIN IDENTIFICATION IN THE SALMONELLAS

by NANCY ATKINSON[1]

(From the Department of Bacteriology, University of Adelaide, South Australia).

(*Accepted for publication 12th November, 1956.*)

The bacteriophage grouping scheme proposed here was gradually developed during work on the lysogenicity and lysis patterns exhibited by a Salmonella serotype when many strains were tested among themselves. In the two bacteriophage grouping schemes published so far, one for S. *adelaide* (Atkinson, 1955) and the other for S. *bovis-morbificans* (Atkinson, 1956) the groups were determined by both lysis and lysogenicity patterns using selected phages and indicator strains. However, the results obtained with S. *bovis-morbificans* suggested that lysogenicity pattern alone was adequate for the routine grouping of a strain. The present paper therefore proposes a modified bacteriophage grouping scheme based on lysogenicity and applicable to many different Salmonella serotypes. Like other schemes involving bacteriophage, the bacteriophage grouping scheme aimed at exact identification of a Salmonella strain of a given serotype. The bacteriophage grouping scheme suggested here is not intended to replace any existing schemes, but should prove useful for serotypes for which no scheme of strain identification at present exists.

Other Schemes Using Bacteriophage for Exact Strain Identification of a Salmonella Serotype.

A number of these schemes already exist and have been developed through two different approaches. The first approach, by which the strain was recognised as belonging to a "phage type", depended upon the sensitivity of the strain to lysis by a selected set of phages, usually called typing phages. The second approach depended upon the lysogenicity of the strain and the identification of the carried phage. Though these two approaches appeared different they were in fact related, as many of the phage types were shown to depend upon carried phage. In such phage types, the carried phages were then called natural type determining phages.

---

[1] This work was assisted by a grant from the National Health and Medical Research Council, Canberra.

Schemes developed from the first approach belonged to two distinct categories depending upon the nature of the typing phages. Typing schemes of the first category depended upon specific typing phages which were all derived from a single phage by adaptation and lysed only strains of the corresponding phage type. In typing schemes of the second category, non-specific typing phages (usually anti-O phages) of heterogeneous origin were used and the phage types recognised by their lytic reactions. Typing schemes of one or other of these two categories have been developed for several Salmonella serotypes and are briefly reviewed in the following sections under the heading of the Salmonella serotype concerned.

S. typhi.

The Vi-phage typing scheme for *S. typhi*, developed by Craigie and Yen (1938) and extended by Craigie and Felix (1947) belonged to the first category. It depended upon a series of typing phages all developed by adaptation of the original Vi-phage II of Craigie and Yen (1938). This phage, at the test dilution, lysed only certain strains of *S. typhi* containing Vi antigen, but could be adapted to lyse other Vi strains not previously attacked. The adapted phage then lysed these strains but not those originally attacked. Many different adaptations of Vi phage II were produced and used to differentiate strains of *S. typhi* into Vi phage types which appeared to be stable. Thus exact strain identification of *S. typhi* became possible and proved most valuable in investigations of outbreaks of typhoid fever. Anderson and Felix (1953 a and b) and Anderson and Fraser (1955) showed that two factors were often involved in the adaptation of Vi phage II to a strain of *S. typhi*. One factor was concerned with the bacterial strain itself and the other was the natural Vi type determining phage carried by the strain. By treating certain non-lysogenic type strains with these phages (which were not Vi phages) recognised Vi phage types of *S. typhi* were artificially produced. Some strains, however, failed to yield these natural phages and apparently contained some other factor responsible for adaptation of Vi phage II and thus for determination of Vi phage type. The Vi phage typing scheme for *S. typhi* therefore depended upon specific typing phages all derived from a single parent phage, and the Vi phage type was distinguished by its lysis with the corresponding typing phage and often was determined by carriage of non-Vi type determining phages. This scheme is used internationally and no alternative has been proposed.

S. paratyphi B.

Felix and Callow (1943, 1951) attempted to develop a Vi phage typing scheme for *S. paratyphi* B similar to that existing for *S. typhi*. The phages used were called *S. paratyphi* B Vi phages as they appeared to act specifically upon certain strains of *S. paratyphi* B containing the B Vi antigen but failed to lyse 0-variants. The B Vi antigen was not comparable, however, with the *S. typhi* Vi antigen as it was subsequently identified with 0 antigen 5 of the Kauffmann-White schema. This antigen was not specific for *S. paratyphi* B but was present in many other members of 0 group B. Felix and Callow (1951) found the *S. paratyphi* B Vi phage typing scheme difficult to develop as the original B Vi phage (called Type I) could not easily be adapted to the many untypable strains encountered during attempts to type *S. paratyphi* B from many outbreaks in England. They therefore introduced other phages, not derived from Type I but still called *S. paratyphi* B Vi phages, to allow typing of most of the previously untypable strains. Their scheme no longer resembled that for *S. typhi* as their phages were not all derived from a single parent phage. Furthermore, by serological investigation, they showed that certain B Vi typing phages believed to be adaptations of a single parent phage were unrelated to it serologically and were unlikely to be true adaptations. They also showed that all the type strains of *S. paratyphi* B were lysogenic and some of the carried phages were identical with some of the typing phages. The carried phages were often type determining as strains of a given Vi phage type usually possessed the same natural phage. The *S. paratyphi* B Vi phage typing scheme of Felix and Callow was thus shown to depart from the principles of the Vi phage typing of *S. typhi* and was actually a scheme of the second category using

non-specific typing phages of heterogeneous origin. Later Felix (1955) recognised his B Vi phages as non-specific anti-0 phages. However, the reliability of the Felix and Callow method of phage typing of *S. paratyphi* B had been shown in investigations of numerous outbreaks and it was recommended for international use (Felix, 1953).

Hamon and Nicolle (1951), Nicolle and Hamon (1951) and Nicolle, Hamon and Edlinger (1951) successfully applied the Felix and Callow Vi phage typing scheme to strains of *S. paratyphi* B isolated in France. They showed that strains of many of the phage types were lysogenic and the natural carried phages were often type determining. Treatment of certain type strains with these phages allowed the artificial production of other phage types included in the scheme.

Scholtens (1950, 1952, 1956) applied the Felix and Callow Vi phage typing scheme to strains of *S. paratyphi* B isolated in Holland. In the early work, when five typing phages were provided by Felix and Callow, only 15 p.c. of Dutch strains were typable. Investigations were therefore carried out on the untyped Dutch strains and on the Felix and Callow type strains, all of which proved to be lysogenic. New types were distinguished among the Dutch strains on carriage of certain phages and sensitivity to other phages. After further work on representative strains of all his types, Scholtens suggested a "natural grouping" of strains of *S. paratyphi* B on their reactions to some of the Felix and Callow typing phages and to phages which they generated in mixed culture with other types of *S. paratyphi* B. The groups thus determined were then divided into types on carriage of certain natural type determining phages and reactions to these phages, some of which were identical with some of the Felix and Callow typing phages. The scheme was rather complicated and the generation of phages in mixed cultures during 7 days incubation was an unusual procedure. Nevertheless, by these methods he was able to type most of the Dutch strains. Some of his types were identical with some of the Felix and Callow types.

Thus for strain identification of *S. paratyphi* B the Felix and Callow phage (originally called B Vi phage) typing scheme, depending upon lysis with a set of non-specific anti-0 phages, was the internationally recommended method. On account of the early failure of this scheme in Holland, another scheme was devised by Scholtens and depended partly on phages from the Felix and Callow scheme and partly upon lysogenicity. It was successful in typing Dutch strains and some of its types were identical with the later Felix and Callow types. It offered an alternative though rather more cumbersome method. Both these schemes have shown their worth in epidemiological investigations. As with the *S. typhi* Vi phage types, some of the *S. paratyphi* B phage types depended upon natural type determining carried phage.

S. typhi-murium.

Lilleengen (1948) proposed a phage typing scheme of the second category for *S. typhi-murium*. The typing phages were frankly non-specific anti-0 phages of heterogeneous origin but they allowed sub-division of strains of *S. typhi-murium* into phage types which were shown to be epidemiologically significant.

Felix and Callow (1943) reported the isolation of at least one specific *S. typhi-murium* Vi phage lysing strains of *S. typhi-murium* presumed to contain a Vi antigen, but not lysing 0-variants of *S. typhi-murium* or strains of *S. paratyphi* B. They developed a Vi phage typing scheme for *S. typhi-murium* which was used in England for many years and gave results of epidemiological value. This scheme was not extensively described until Felix (1955) summarised many of the findings. The original Vi phages had not proved adaptable to untyped strains and therefore other typing phages of various origins had to be introduced. On this account the typing scheme was not comparable with the Vi phage typing scheme for *S. typhi* and the typing phages were, in fact, non-specific anti-0 phages. Nevertheless epidemiological relationships were indicated and the typing scheme was used successfully to investigate many outbreaks in England. In spite of the success of this typing scheme, an attempt was made to

develop a new phage typing scheme for *S. typhi-murium* in which all the typing phages were derived by adaptation from a single parent phage and thus conformed more closely to the Vi phages used for typing *S. typhi*. After much labour a set of adapted typing phages of common parentage was obtained and used to type a series of strains of *S. typhi-murium* previously typed by the original method. A totally different typing of these strains resulted and cut across their typing by the original method. The lytic reactions of a strain often correlated with the presence of natural phages. Epidemiological relationships were indicated equally well by the original and the new method. Felix therefore stated that his earlier contention (Felix, 1951) that Lilleengen's typing scheme for *S. typhi-murium* was less valid than the Felix and Callow method, was premature. He had based this contention on the demonstration that Lilleengen's scheme, which used non-specific anti-0 phages, cut across the typings of the Felix and Callow scheme which was more valid as it used Vi phages specific for *S. typhi-murium*. He now recognised the Vi phages as non-specific anti-0 phages and had shown that more than one valid typing scheme could be developed with such phages. Therefore, three typing schemes, two from Felix and one from Lilleengen, existed for *S. typhi-murium*. Each was equally capable of giving typings of epidemiological value. One belonged to the first category using typing phages from a single parent phage and two belonged to the second category using heterogeneous phages.

S. dublin, S. enteritidis, S. pullorum, S. gallinarum and S. thompson.

For all these Salmonellas, phage typing schemes of the second category have been developed. The phage types depended upon lytic reactions with a set of non-specific anti-0 typing phages. Thus Lilleengen (1950, 1952) demonstrated phage types of *S. dublin*, *S. enteritidis*, *S. pullorum* and *S. gallinarum*. Williams Smith (1951a) also demonstrated phage types for *S. dublin* which were different from those of Lilleengen. These two typing schemes differed in the number of typing phages used and in the number of phage types recognised. Williams Smith (1951b) further described phage typing of *S. thompson* and noted that *S. cholerae-suis* by similar tests provided little strain differentiation and was probably unsuitable for phage typing. He also showed, by the comparison of natural strains of various phage types and strains artificially treated with phages from these types, that acquired phage resistance, whether accompanied by lysogenicity or not, was often responsible for a strain being placed in a particular phage type.

Thus for the seven Salmonella serotypes *S. paratyphi* B, *S. typhi-murium*, *S. dublin*, *S. enteritidis*, *S. pullorum*, *S. gallinarum* and *S. thompson*, phage typing schemes of the second category, depending on non-specific typing phages, have been proposed. For *S. typhi*, however, the internationally recognised Vi phage typing scheme belonged to the first category and used specific adaptations of a single parent phage. All these schemes gave typings of epidemiological value and many of the phage types were shown to be determined by natural carried phage.

Schemes using carried phage for strain identification.

The second approach to exact strain identification in the Salmonellas through recognition of their carried phages was first suggested by Burnet (1932), but he did not follow it up. He was impressed with the permanence of the lysogenic state and considered that strains of a Salmonella serotype could be identified and related to each other by the carriage of particular kinds of phage which were permanent characters of the strains. Boyd (1950) independently had the same idea (which he later discovered had previously occurred to Burnet) and applied it to strain identification of *S. typhi-murium*. Preliminary work involved the isolation and characterisation of the carried phages, called symbiotic phages, of a large number of strains of *S. typhi-murium*. Among all these symbiotic phages, only eight different types were found and sub-divided into the A and B groups of Burnet (1932). Three types were placed in Group A and five in Group B. These eight types covered many of the common symbiotic

phages of *S. typhi-murium*. Boyd was then in a position to plan a field trial of the value of strain identification by recognition of the symbiotic phage. Boyd, Parker and Mair (1951) therefore collected numerous strains of *S. typhi-murium* of varied or related origin and examined them for the type of phage carried. Three new types of phage were found, one in Group A and two in Group B. The routine testing for the presence and type of symbiotic phage was a little cumbersome but nevertheless workable. As far as could be judged from the strains available, the results correlated well with the epidemiological data. The authors stressed that their method of strain identification was not to be confused with the Felix and Callow Vi phage typing scheme for *S. typhi-murium* nor was their new method put forward to supplant existing schemes. To avoid any confusion in terms, the word "type" was not used but the strains were identified by a Mark signifying the type of carried phage. Thus strains of *S. typhi-murium* carrying phage type A1 were identified by the Mark A1. Very few strains were marked "phage undetected" and the authors considered that by suitable methods all strains of *S. typhi-murium* would yield phage.

Investigation of carried phage also contributed to the definition of the groups and types of *S. paratyphi* B suggested by Scholtens (1956) and mentioned earlier. The generation of certain phages in mixed cultures over a week and also the production of certain natural phages readily detected and identified in young cultures partly served to identify groups and types. However, this scheme also used typing phages and differed from Boyd's method of marking *S. typhi-murium* in not attempting to identify all the carried phages nor using them all in designating groups or types. Thus Boyd's method of identification of strains of *S. typhi-murium* by identification of carried phage was unique and had not been applied to any other Salmonella serotype. Boyd pointed out, however, that it was applicable to any lysogenic Salmonella serotype.

The Proposed Scheme of Bacteriophage Grouping.

From the foregoing it is obvious that different workers, by their arbitrary selection of typing phages, were able to propose totally different phage typing schemes for the same Salmonella serotype. Each scheme apparently gave results of epidemiological significance. Such a multiplicity of schemes is, however, undesirable and can lead to confusion and controversy unless a particular scheme is adopted by international agreement.

In contrast to the phage typing schemes, Boyd's scheme of marking strains by their symbiotic phage seemed more satisfactory as it depended upon the permanent character of lysogenicity and was therefore likely to give the same grouping of strains in the hands of all workers. For *S. typhi-murium*, Boyd, Parker and Mair (1951) provided evidence that their scheme of marking gave results of epidemiological significance. The techniques of marking were not simple, however, and with *S. typhi-murium* at least 11 different types of symbiotic phage had to be recognised. The authors also pointed out that the method of obtaining the symbiotic phage was rather laborious. They suggested, however, that if the methods could be simplified, their scheme of strain identification would have to be seriously considered as an alternative to already existing schemes.

The bacteriophage grouping scheme proposed here was developed from extensive work on the lysogenicity and lysis patterns of several Salmonella serotypes which was recorded in the earlier papers of the present series. It was an

attempt at simplification of Boyd's scheme of marking a strain by its symbiotic phage. In the proposed scheme the symbiotic phage of a strain was not actually identified, but was recognised by its range of activity in lysogenicity tests on a series of indicator strains representing the established phage groups. The indicator strains carried different phages which had previously been characterised as numbered types. Each indicator strain and thus each phage group therefore corresponded to one of Boyd's Marks but was distinguished, not by direct identification of its symbiotic phage, but by its typical lysogenicity pattern on the indicator strains of the existing groups. This pattern was, of course, a direct reflection of the carried phage. In the examples of grouping schemes in Table 1 these patterns are given in the horizontal rows.

In practice, therefore, the grouping of a strain was determined by its lysogenicity pattern in lysogenicity tests involving the test strain with each of the indicator strains. These lysogenicity tests were done by growing the test strain and the indicator strain together in mixed culture on agar overnight at 37° C., extracting the mixed growth in a little broth, centrifuging the extract, and testing for lytic activity on the two strains from which it came. The tests for lytic activity were done by the method of Craigie and Yen (1938). If the extract lysed the indicator strain the lysogenicity test was recorded as positive (+), if no lysis occurred it was recorded as negative (−). From the pattern of results of lysogenicity tests on all the indicator strains the grouping of a test strain was determined. If it failed to give a recognisable lysogenicity pattern, the strain was recorded as ungrouped and was eventually examined with other ungrouped strains for the establishment of new groups. The lysogenicity tests also gave the lysogenicity pattern of the indicator strains on the test strain. This pattern was not used in routine grouping but can be seen in the vertical rows in the examples of grouping schemes in Table 1. In the routine lysogenicity tests it was always determined as it involved little extra work (one lysis test with the already prepared extract) and provided additional information which was sometimes useful in deciding the grouping of doubtful strains. To be accepted for a group a strain must primarily show no lysogenic activity with the corresponding indicator strain, and in doubtful groupings the lysogenicity pattern of the indicator strains on the test strain, though not usually considered in grouping, might be decisive. Failure to lyse any of the indicator strains (apparent absence of lysogenicity) was recognised as a typical group pattern in some Salmonella serotypes (see Table 1, S. *waycross* Atherton group and S. *adelaide*, group 4). Boyd, Parker and Mair (1951) considered absence of symbiotic phage to be just as valid a Mark as the presence of a symbiotic phage, especially in those Salmonella serotypes in which many strains were lysogenic.

Examples of bacteriophage grouping schemes of the kind proposed here are given in Table 1 for S. *waycross*, S. *adelaide* and S. *bovis-morbificans*. For these three serotypes, indicator strains, carrying defined phage were already established

and assigned to phage groups on lysis and lysogenicity patterns and type of phage carried (Atkinson and Carter, 1953; Atkinson, 1955, 1956a). These phage groups have now been recognised and defined simply by their lysogenicity patterns according to the proposed bacteriophage grouping scheme. In Table 1, the typical lysogenicity pattern of each group is represented by the reactions of its indicator strain shown in the horizontal rows.

TABLE 1.

Bacteriophage grouping schemes proposed for strain identification of S. waycross, S. adelaide *and* S. bovis-morbificans.

Proposed phage group	Indicator strain tested	Lysogenicity test on indicator strains					
S. waycross		Currie (Atherton group)			Cronin (Group 1)		
Atherton group	Currie	−			−		
Group 1	Cronin	+			−		
S. adelaide		2 (group 1a)	20 (group 1b	37 (group 2a)	273 (group 2c)	14 (group 3)	Cey 4 (group 4)
Group 1a	2	−	±	+	+	+	+
Group 1b	20	±	−	+	+	+	+
Group 2a	37	+	+	−	−	+	+
Group 2c	273	+	+	+	−	+	+
Group 3	14	−	−	−	−	−	+
Group 4	Cey 4	−	−	−	−	−	−
S. bovis-morbificans		17 (group 2)	45 (group 3)	18 (group 4)	14 (group 5)	8 (group 6)	
Group 2	17	−	±	+	+	+	
Group 3	45	+	−	+	+	+	
Group 4	18	−	−	−	−	+	
Group 5	14	+	+	+	−	+	
Group 6	8	+	+	+	+	−	

The grouping schemes in Table 1 for the three serotypes differed from each other in the number of recognised groups and thus of indicator strains. The number of groups obtainable depended upon the number of different types of carried phage. S. *waycross* provided only one type of carried phage and the grouping was simply into the lysogenic strains (group 1) and the non-lysogenic strains (Atherton group). S. *adelaide* strains carried several different types of phage or were non-lysogenic. The grouping was therefore into a non-lysogenic group 4 and the five lysogenic groups, 1a, 1b, 2a, 2c and 3, each carrying different types of phage and distinguished by their lysogenicity patterns. A few strains were ungrouped and appeared to carry new types of phage. These strains, when adequately investigated, will probably be placed in new groups. S. *bovis-*

morbificans strains were all lysogenic and carried many different types of phage. The grouping was therefore into the five lysogenic groups 2, 3, 4, 5 and 6, carrying different types of phage and distinguished by their lysogenicity patterns. Many strains failed to fit into any of these groups. They carried several different types of phage and will eventually provide several additional groups.

The phage grouping scheme for S. *waycross* was applied to 33 strains of which 29 came from a single outbreak of diarrhoea in Atherton, Queensland, described by Ferris, Hertzberg and Atkinson (1945). All these strains were placed in the non-lysogenic Atherton group but two strains from unrelated cases were lysogenic and were placed in group 1. By this grouping, the related Atherton strains were all placed together and distinguished from the two unrelated strains. The grouping thus appeared to be epidemiologically valid. Two strains isolated much later from Queensland lizards (Lee and Mackerras, 1955) also belonged to the Atherton group and suggested that the earlier outbreak might have originated from lizards. The grouping of the S. *waycross* strains correlated with dulcitol fermentation. All strains of the Atherton group fermented dulcitol rapidly but the two strains of group 1 failed to ferment dulcitol (Atkinson, Woodroofe and Macbeth, 1950). The epidemiological value of the grouping of strains of S. *adelaide* could not easily be assessed as the available strains were mainly of heterogeneous origin. The grouping of strains of S. *bovis-morbificans* agreed well with the epidemiological evidence. Among 153 strains investigated (Atkinson and Bullas, 1956a) 95 came from Brisbane (Queensland) in 1947 when an epidemic of gastro-enteritis occurred (Mackerras and Mackerras, 1949). Of these 95 Brisbane strains, 80, obviously connected with the epidemic, were placed in group 6. Otherwise, very few group 6 strains were found. This grouping of S. *bovis-morbificans* therefore placed many related strains together and seemed to be epidemiologically significant.

For comparison with the proposed phage grouping scheme, a phage typing scheme and a scheme based on Boyd's marking by symbiotic phage were also developed for the three serotypes for which phage grouping schemes were given in Table 1. These schemes are given in Table 2. In the phage typing schemes, the arbitrarily selected phages were all symbiotic phages isolated from lysogenic strains of the serotype being tested. In the scheme following Boyd's marking by type of carried phage, the phages of the lysogenic indicator strains were isolated on strains of the same serotype and characterised by various reactions (Atkinson, 1956b; Atkinson and Bullas, 1956b and c; Atkinson and Geytenbeek, 1953).

The groups already established in the phage grouping schemes in Table 1 were shown to give typical reactions and thus to be recognisable by either of the other two schemes in Table 2. In the phage typing scheme using the arbitrarily selected phages, each group gave a typical lysis pattern. By the scheme based on Boyd's marking by symbiotic phage, each group was also clearly distinguished by the type of phage carried. Some groups carried two phages, one of

TABLE 2.

Two other alternative schemes (phage typing and Boyd's scheme of marking by symbiotic phage) for strain identification of S. waycross, S. adelaide *and* S. bovis-morbificans

Proposed phage group	Indicator strain tested	Phage typing scheme Lysis with phage					Boyd's marking scheme. Type of phage carried
S. waycross		W Cr (type 1)					
Atherton Group	Currie	+					none detected
Group 1	Cronin	−					type 1
S. adelaide		Ad 2 (type 1a)	Ad 20 (type 1b)	Ad 37H (type 2a)	Ad 273 (type 2c)	Ad 14 (type 3)	
Group 1a	2	−	+	+	+	−	types 1a and 3
Group 1b	20	+	−	+	+	−	types 1b and 3
Group 2a	37	+	+	−	+	−	types 2a and 3
Group 2c	273	+	+	−	−	−	types 2c and 3
Group 3	14	+	+	+	+	−	type 3
Group 4	Cey 4	+	+	+	+	+	none detected
S. bovis-morbificans		BM17/18 (type 2)	BM45/18 (type 3)	BM14/8 (type 4)	BM14/18 (type 5)	BM8/14 (type 6)	
Group 2	17	−	+	−	+	+	type 2 and an unidentified type
Group 3	45	−	−	−	+	−	type 3 and an unidentified type
Group 4	18	+	+	−	+	+	type 4
Group 5	14	+	+	−	−	+	types 4 and 5
Group 6	8	+	−	+	−	−	types 5 and 6

which was the same in all groups, which were then distinguished by carriage of a typical and distinctive second phage (see S. *adelaide* groups 1a, 1b, 2a and 2c, S. *bovis-morbificans* groups 5 and 6, Table 2). As the proposed phage grouping scheme in Table 1 and the scheme of marking by carried phage in Table 2 both depended upon the type of carried phage, both schemes gave identical groupings. The phage typing scheme in Table 2, depending upon the lytic activity of its phages, also gave identical groupings on account of the arbitrary selection of the carried phages as typing phages. Typing phages arbitrarily selected from other sources would probably give different results. The three schemes, as presented in Tables 1 and 2, were, however, identical in their groupings and any one of them should therefore be suitable for routine use in strain identification.

The proposed phage grouping scheme, however, showed several distinct advantages over the other two schemes. No maintenance of typing phages was required (an advantage over the phage typing scheme) nor was exact identification of the carried phage necessary (an advantage over Boyd's scheme of

marking by symbiotic phage). The only maintenance work involved was the preservation and care of the indicator strains which were kept dried and in agar stabs. The dried cultures were used only for replacements and all working cultures came from the stock agar stabs which were sub-cultured every six months. The indicator strains in Table 1 were all kept in agar stabs in this way for four to eight years and showed no change. The maintenance of the indicator strains therefore involved little work and required no special skill or knowledge. By contrast, the maintenance of the typing phages of a phage typing scheme, required considerable skill and experience and involved much work, which increased as the number of typing phages increased. Some of the typing phages were weak and unstable and required frequent propagation and re-isolation (e.g. phages Ad 20 and BM 17/18). The phage preparations used in routine work had to be regularly checked for lytic spectrum and titrated for test dilution. The stock phage preparations from which they came had to be propagated every one to six months depending on stability. The newly propagated phage stocks had to be titrated and checked for lytic spectrum. Serial propagation of some of the *S. adelaide* typing phages resulted in the appearance of host range mutants which made the preparations unsatisfactory for typing. If such mutants were detected, the original phage had to be re-isolated. In all phage typing schemes, such as the Vi phage typing of *S. typhi* or the phage typing of *Staph. aureus* (Williams and Rippon, 1952) the care and maintenance of the typing phages is a major part of the work and requires special knowledge of the phages. In the proposed phage grouping scheme all this maintenance work was avoided. Comparing the two schemes, however, for the work of actual routine testing, the phage typing scheme was simpler. It required only the spotting of each typing phage on the test strain, the number of spots depending on the number of phages, whereas the phage grouping scheme required the preparation of an extract from the mixed growth of each indicator strain with the test strain, followed by spotting the extract on its indicator and test strains. The number of extracts depended upon the number of indicator strains. Taking into account, however, the maintenance work as well as the routine testing in the two schemes, the proposed phage grouping scheme was much simpler and required much less time, skill and experience for its successful application. For these reasons it should be more suitable for general routine use in other laboratories.

By comparison with the proposed phage grouping scheme, the schemes in Table 2 based on Boyd's method of marking by the type of symbiotic phage, were more complicated. The carried phages isolated from the indicator strains were characterised by various tests and allotted to numbered types to facilitate their future recognition. A collection of representative phages was maintained for reference and formed a nucleus of designated phages similar to Boyd's collection of *S. typhi-murium* phages. The actual tests for marking a strain by its symbiotic phage, according to the schemes in Table 2, were neither simple nor easy and

required intimate knowledge of the phages. Several of the S. *adelaide* phages produced similar plaques, were similar in heat sensitivity and had to be distinguished by host range and serology. The S. *bovis-morbificans* phages were divisible on heat resistance but several of the heat sensitive phages produced similar plaques and their isolation and identification were complicated by the necessity of using lysogenic propagating strains whose phages frequently propagated at the same time as the phage under investigation. More complicated testing than that used by Boyd, Parker and Mair (1951) for S. *typhi-murium*, was therefore necessary to recognise the carried phages of S. *adelaide* and S. *bovis-morbificans*. Compared with these schemes of marking by the type of symbiotic phage, the proposed phage grouping schemes offered a much simpler way of recognising the type of lysogenicity of a strain by its characteristic lysogenicity pattern. No isolation or identification of phage was required and relatively inexperienced workers could carry out, read and interpret the tests. Like Boyd's marking scheme, the proposed phage grouping scheme was based on the stable characteristic of lysogenicity and should give an absolute grouping. Its indicator strains were not arbitrarily selected but were picked for their known phage content. Each group actually represented one of Boyd's Marks.

On account of its greater simplicity, the proposed phage grouping scheme was therefore considered preferable to either the phage typing scheme or Boyd's scheme of marking by type of symbiotic phage. It appeared to have potential value in epidemiological investigations and only by further use can its actual value be established.

The proposed phage grouping scheme could be applied not only to other lysogenic Salmonellas, but also to any other lysogenic species of bacteria. It should be easily applicable to S. *typhi-murium* by using strains representing all Boyd's Marks as indicator strains. It might also be applicable to some species, for which a phage typing scheme related to lysogenicity had been developed. The strains of the established phage types would then be used as indicator strains. However, little correlation might be found with phage types as Anderson and Felix (1953b) stated that "the recently proposed method of typing bacteria by identifying the phages they carry (referring to Boyd's marking of S. *typhi-murium*) is unsuitable for application to S. *typhi*."

Preliminary work on S. *potsdam*, S. *senftenberg*, S. *muenchen* and S. *derby* showed that strains of all these serotypes were frequently lysogenic and carried a variety of phages. They were therefore suitable for development of phage grouping schemes. For S. *potsdam* a rudimentary scheme has been proposed and a description of some of the carried phages given (Atkinson and Bullas, 1956d and e).

The indicator strains of the schemes given in Tables 1 and 2 are available for any worker wishing to use them. The typical lysogenicity pattern of each group can be seen by testing each indicator strain against all the indicator strains

for that serotype. A worker may thus become familiar with the group patterns before actually attempting phage grouping. As new groups are recognised or new schemes developed, more indicator strains will become available. From this work, a collection of strains of various Salmonella serotypes of known lysogenic status is being built up in this laboratory. Not only are these strains valuable as indicators in phage grouping but they also form a source of designated types of Salmonella phage which can be obtained from them by suitable manipulation. These phages, it is hoped, will eventually form a large enough collection to provide data upon which an extended classification of Salmonella phages, based on the work of Burnet and Boyd, can be developed.

The proposed phage grouping scheme is open to several criticisms. In the actual performance of the test the extracts tested for lysis on the indicator and test strains were not bacteriologically sterile. Contamination of the test spot of the extract was therefore possible with either the test strain or the indicator strain from which it came. In practice, however, the extracts were well centrifuged and very few false positive or false negative results were obtained. Any treatment of the extract to produce bacteriological sterility, such as heating or filtration, risked the loss of some phage and therefore no treatment other than centrifuging was recommended. A more serious criticism could be based on the possibility that the phage grouping of a strain might change by either loss of a phage or acquisition of a new phage during its passage from one place to another. Such a change would, of course, render phage grouping useless for tracing strains. However, the same criticism is applicable to the scheme of marking by symbiotic phage and to a more limited extent to phage typing schemes. Boyd, Parker and Mair (1951) realised this possibility which could affect their marking of *S. typhimurium*. Boyd (1950) considered carriage of a particular phage to be a permanent character. However, one of the few examples of loss of lysogenicity was described by Anderson and Felix (1953b) for *S. typhi* Vi type F2 in which the lysogenic state was unstable and from which phage-free organisms of Vi type F1 were thrown off after long periods of storage. The acquisition of phage during the wanderings of a strain, especially if non-lysogenic, seemed much more likely than loss of phage. Boyd, Parker and Mair (1951), however, dismissed this possibility as very unlikely and Anderson and Felix (1953b) pointed out "the fact that world-wide experience with the Vi phage typing technique over many years had proved its epidemiological reliability suggests that the chances of the typhoid bacillus encountering type-changing phages under natural conditions are remote". Such changes are not impossible, however, as suitable strains have been changed in their phage type or their phage group by treatment in the laboratory with certain phages which they apparently rarely met in natural conditions. No evidence for any change was found in the phage grouping of strains of *S. bovismorbificans* from the Brisbane epidemic. The majority belonged to phage group 6 which carried two phages, types 5 and 6. Some of the strains had probably been

through a fairly extensive and varied journey as they came from mice and cockroaches thought to be innocent victims and found late in the epidemic. But they still showed the typical lysogenicity pattern of phage group 6 and carried types 5 and 6 phage. A few strains which appeared to be connected with the epidemic belonged to phage group 3 with a different lysogenicity pattern from phage group 6 and carrying type 3 phage and another unidentified phage. These strains obviously represented sporadic infections and could not possibly have been derived from phage group 6. In fact, with the five phage groups recognised so far for *S. bovis-morbificans*, only one group transformation would be possible and could occur from phage group 4 carrying type 4 phage to phage group 5 carrying types 4 and 5 phage. On account of their existing phages none of the other phage groups could be derived from each other. The change of strains of phage group 4 to phage group 5 seemed most unlikely under natural conditions, as phage group 5 was very rare and phage group 4 was not uncommon. In *S. adelaide*, however, all phage groups could be derived from the non-lysogenic group 4 and some phage groups could be derived from group 3. Thus phage groups 1a, 1b, 2a and 2c, all carrying type 3 phage and another phage could each be derived from phage group 3 carrying type 3 phage or phage group 4 carrying no phage. In the laboratory each of these groups was made artificially by treatment of a group 3 strain with the corresponding phage type 1a, 1b, 2a or 2c. From a group 4 strain, group 3 was easily prepared by treatment with type 3 phage, and this artificially produced group 3 strain could then give rise to all the other groups by treatment with the corresponding phage. In Australia phage group 3 was by far the commonest and thus seemed unlikely under natural conditions to meet phage of type 1a, 1b, 2a or 2c which could transform it into another group. The evidence so far available on the stability of the phage groups of the proposed phage grouping schemes under natural conditions favours the probability that they seldom meet phages capable of transforming them into other groups and are therefore stable and should be useful for tracing strains in epidemics.

SUMMARY.

A bacteriophage grouping scheme depending on lysogenicity was suggested for strain identification in the Salmonellas. For simplicity of method and stability of grouping the scheme was considered to be at least equal to and probably better than phage typing schemes or schemes of marking by type of symbiotic phage. The phage group of a strain was determined by its lysogenicity pattern on the indicator strains representing the recognised groups. For *S. waycross*, *S. adelaide* and *S. bovis-morbificans*, phage grouping schemes were devised and allowed grouping of at least the majority of strains tested. Evidence was provided for the stability of the phage groups and their potential value in epidemiological investigations. The proposed scheme for bacteriophage grouping for exact strain identification should be applicable not only to lysogenic Salmonella serotypes but also to any other lysogenic species of bacteria.

REFERENCES.

Anderson, E. S., and Felix, A. (1953a): J. gen. Microbiol., 8, p. 408.
Anderson, E. S., and Felix, A. (1953b): *Ibid.*, 9, p. 65.
Anderson, E. S., and Fraser, A. (1955): *Ibid.*, 13, p. 519.
Atkinson, N. (1955): Austral. J. exp. Biol., 33, p. 371.
Atkinson, N. (1956a): *Ibid.*, 34, p. 231.
Atkinson, N. (1956b): *Ibid.*, 34, p. 361.
Atkinson, N., and Bullas, L. R. (1956a): *Ibid.*, 34, p. 349.
Atkinson, N., and Bullas, L. R. (1956b): *Ibid.*, 34, p. 461.
Atkinson, N., and Bullas, L. R. (1956c): *Ibid.*, 34, p. 27.
Atkinson, N., and Bullas, L. R. (1956d and e): *Ibid.*, 34, p. 445 and p. 455.
Atkinson, N., and Carter, M. C. (1953): *Ibid.*, 31, p. 591.
Atkinson, N., and Geytenbeek, H. (1953): *Ibid.*, 31, p. 441.
Atkinson, N., Woodroofe, G. M., and Macbeth, A. M. (1950): *Ibid.*, 28, p. 367.
Boyd, J. S. K. (1950): J. Path. Bact., 42, p. 501.
Boyd, J. S. K., Parker, M. T., and Mair, N. S. (1951): J. Hyg., 49, p. 442.
Burnet, F. M. (1932): J. Path. Bact., 35, p. 851.
Craigie, J., and Felix, A. (1947): Lancet, I, p. 823.
Craigie, J., and Yen, C. H. (1938): Canad. pub. Health J., 29, p. 448.
Felix, A. (1951): Brit. med. Bull., 7, p. 153.
Felix, A. (1953): Sixth Int. Congr. Microbiol., Rome.
Felix, A. (1955): J. gen. Microbiol., 14, p. 208.
Felix, A., and Callow, B. (1943): Brit. med. J., II, p. 127.
Felix, A., and Callow, B. (1951): Lancet, II, p. 10.
Ferris, A. A., Hertzberg, R., and Atkinson, N. (1945): Med. J. Austral., II, p. 368.
Hamon, Y., and Nicolle, P. (1951): Ann. Inst. Past., 80, p. 496.
Lee, P. E., and Mackerras, I. M. (1955): Austral. J. exp. Biol., 33, p. 117.
Lilleengen, K. (1948): Acta path. microbiol. Scand. Suppl. 77.
Lilleengen, K. (1950): *Ibid.*, 27, p. 625.
Lilleengen, K. (1952): *Ibid.*, 30, p. 194.
Mackerras, I. M., and Mackerras, M. J. (1949): J. Hyg., 47, p. 166.
Nicolle, P., and Hamon, Y. (1951): Ann. Inst. Past., 81, p. 614.
Nicolle, P., Hamon, Y., and Edlinger, E. (1951): Ann. Inst. Past., 80, p. 479.
Scholtens, R. T. (1950): Ant. v. Leeuw., 16, p. 256.
Scholtens, R. T. (1952): *Ibid.*, 18, p. 251.
Scholtens, R. T. (1956): *Ibid.*, 22, p. 65.
Williams, R. E. O., and Rippon, J. E. (1952): J. Hyg., 50, p. 320.
Williams Smith, H. (1951a): J. gen. Microbiol., 5, p. 919.
Williams Smith, H. (1951b): *Ibid.*, 5, p. 472.

SOME PHARMACOLOGICAL PROPERTIES OF HIMANDRINE, AN ALKALOID DERIVED FROM *HIMANTANDRA BACCATA*

by L. B. COBBIN and R. H. THORP

(From the Department of Pharmacology, the University of Sydney).

(*Accepted for publication, 6th September, 1956.*)

Himantandra is a genus found in New South Wales and Queensland from which many phytochemical substances have been prepared.

A sample of himandrine HCl was supplied by Dr. E. Ritchie of the Department of Organic Chemistry in this University—derived from the bark of *H. baccata.*

Initial experiments upon the anaesthetised cat revealed that the alkaloid caused a depressor response accompanied by bradycardia and it was decided to investigate the pharmacological properties more fully, especially as a similar response is observed following the administration of a large number of phytochemical substances (Krayer and Acheson, 1946; Plummer *et al.*, 1954; Moran *et al.*, 1954) of which the alkaloids of Veratrum and Rauwolfia, for example, have obtained a place in therapeutics.

The alkaloid used in these experiments had the empirical formula $C_{36}H_{37}O_6N.HCl$., m.p. 222° C. with decomposition, and was slightly water soluble. The structural formula is at present unknown. (For the chemistry of the products of *H. baccata* see Brown *et al.*, 1956.)

EXPERIMENTAL.

Toxicity and central nervous actions.

The toxicity of himandrine by intravenous injection was determined in male white mice weighing 18-22 gm. The doses were given in 0·25 ml. distilled water since himandrine is not very soluble in physiological saline. An approximate value for the L.D. 50 of 34 mg./kg. was determined from the log. dose — probit response curve for the twenty-four hour mortality figures shown in Table 1.

The principal toxic signs were disturbances of postural reflexes followed by paralysis of the hindlimbs. Respiration was of a "gasping" nature and the rate was slow.

Death was preceded by symmetrical convulsions.

In order to determine whether himandrine possessed an action on the central nervous system other than toxic convulsions, two groups of male white mice

TABLE 1.

Toxicity of himandrine in mice after intravenous injection.

Dose (mg./kg.)	Mortality
25	0/10
30	1/10
35	6/10
40	8/10
50	10/10

Approximate L.D. 50 = 34 mg./kg.

each weighing between 18 and 22 gm. were placed in activity cages (Cobbin *et al.*, 1955), allowed to settle down to their new environment for one hour, and then one group received himandrine (10 mg./kg. i.p.) and the others an equivalent volume of distilled water (·25 ml.). The results of these experiments were inconclusive; sometimes there was a slight increase in activity of the test group, over the controls, whereas in other experiments the activity in the test group was slightly less than the controls. Never was there a clear-cut stimulation or depression of activity.

Anticonvulsant and sedative actions were further examined by an investigation of the influence of himandrine upon the convulsive dose of leptazol in mice.

The L.D.50 of leptazol was determined by subcutaneous injection in male white mice weighing between 20 and 25 gm. A second group of mice received a sub-cutaneous dose of 20 mg./kg. of himandrine thirty minutes prior to the same range of doses of leptazol as those in group I. A third group received 20 mg./kg. of himandrine alone, which was chosen to be non-toxic. The results are quoted in Table 2 and the L.D.50 values for leptazol alone, and leptazol after himandrine, were determined graphically to be 78 mg./kg. and 82 mg./kg. respectively. In each case the log. dose-response curves were parallel and the difference was not significant. There is, therefore, no evidence of anticonvulsant activity in this experiment.

TABLE 2.

Effect of himandrine upon the L.D. 50 of leptazol in mice.

Dose of leptazol mg./kg.	Mortality	
	Control group	Himandrine-pretreated group
65	3/20	4/20
80	13/20	9/20
95	15/20	14/20
110	20/20	19/20
125	20/20	20/20
L.D. 50	78 mg./kg.	82 mg./kg.

Effects of himandrine on isolated guinea-pig ileum.

Segments of freshly prepared guinea-pig ileum were placed in a 10 ml. organ bath of Tyrode's solution maintained at 37° C. and aerated with 5 p.c. carbon dioxide in oxygen. Doses of himandrine up to 100 μg. alone produced a very slight decrease in tone of the muscle which was reversed by washing, but was not abolished by atropine. The technique of Goldberg and Shapero (1953) was modified to provide a more quantitative comparison of the spasmolytic action of himandrine with papaverine. A standard reproducible spasm of two minutes duration was produced by injecting a quantity of the spasmogenic drug into the bath, and then the gut was washed with fresh Tyrode solution and allowed a two-minute recovery interval before another spasm was induced. At the height of each alternate contraction a measured quantity of himandrine or papaverine was added to the bath and allowed to act for the duration of the two-minute interval. The amount of relaxation produced by the drug was expressed as a percentage of the preceding control spasm, and plotted against the logarithm of the dose of spasmolytic agent. In this way, parallel log. dose-response curves were obtained and the potency of himandrine with respect to papaverine was estimated. A typical set of kymograph records is shown in Fig. 1. The mean potencies of himandrine as a spasmolytic in terms of papaverine are given in Table 3.

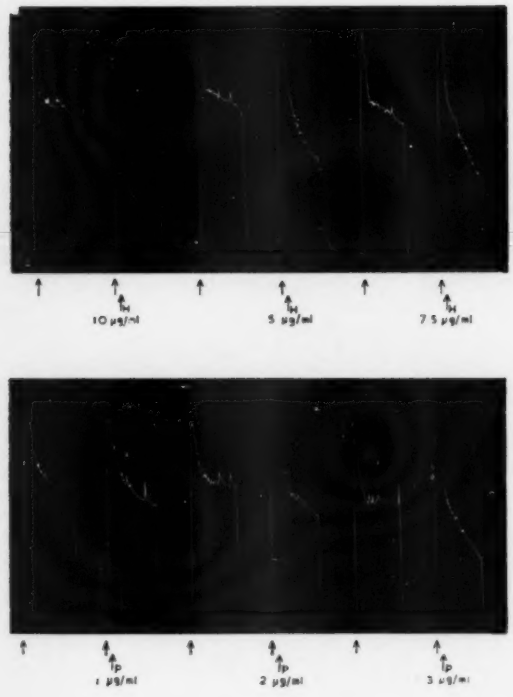

Fig. 1. Record of spasmolytic action of himandrine compared with papaverine on the isolated guinea-pig ileum. Each spasm is of two minutes duration and is induced by 1 μg./ml. of acetyl choline, and is indicated by the upper row of arrows. Alternate spasms are treated with himandrine in the upper record shown by an arrow labelled H and a figure indicating the final concentration of himandrine in the bath. In the lower record the same captions apply with P referring to papaverine.

It was found that carbamyl choline and histamine produced very satisfactory spasms, whereas

those induced by acetylcholine and barium chloride were rather more variable. It was difficult to estimate spasmolytic potency against barium chloride since upon introduction of the spasmolytic, the piece of gut responded by a succession of spontaneous contractions of large amplitude which were removed by washing.

TABLE 3.

A comparison of the spasmolytic activities of himandrine and papaverine on guinea-pig ileum.

Spasm induced by	Potency (Papaverine = 1·0)
Acetylcholine	0·9
Carbamylcholine	0·3
Histamine	0·5
Barium Chloride	2-3

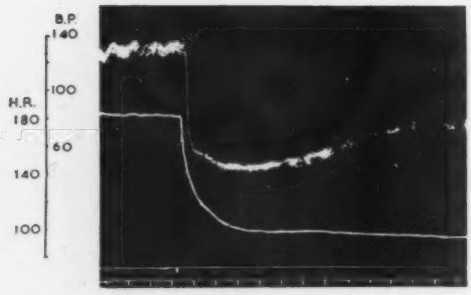

Fig. 2. The effects of himandrine on the left carotid blood pressure and heart rate in the intact cat. Records from top to bottom blood pressure, graph of heart rate per minute (from values obtained with an e.c.g.), signal marker and time marker showing minutes. At the point indicated by the signal 2 mg./kg. himandrine was injected into the right saphenous vein.

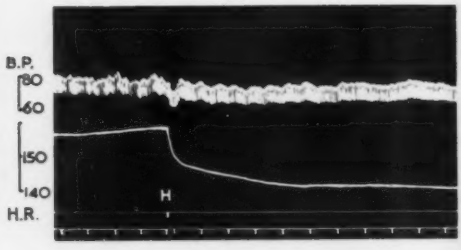

Fig. 3. The effects of himandrine on the left carotid blood pressure and heart rate in the spinal cat. The records from top to bottom as in Fig. 2, and the dose of himandrine was 2 mg./kg.

Effects of himandrine in the intact animal.

Cats anaesthetised with 50 mg./kg. pentobarbitone sodium intraperitoneally were prepared for intravenous injection via the right saphenous vein and for blood pressure recording from the left common carotid artery. Heart rates were determined from an e.c.g. connected as lead II.

Doses of himandrine ranging from 0·2 mg./kg. to 5·0 mg./kg. produced a graded fall in the arterial blood pressure and heart rate. A dose of 2·0 mg./kg. gave a fall in blood pressure of approximately 66 p.c. (Fig. 2), after a latent period of 20-30 seconds, returning to a stable level of approximately 90 p.c. of the pre-injection level in one hour (not shown in Fig. 2). Larger doses pro-

duced a greater fall in blood pressure and the duration of the effect lasted up to thirty minutes. The heart rate was lowered from 182 per minute to 97 just after the minimum of the blood pressure trace — a fall of the order of 47 p.c. Unlike the blood pressure response, the heart rate failed to return to the pre-injection level. Neither response was blocked by atropine or by bilateral vagotomy.

It was found that the administration of small doses of adrenaline (1-2 μg.) will momentarily antagonise both the depressor and bradycardiac responses resulting from administration of himandrine. However, the responses returned to their original recovery course as though the adrenaline had not been administered. After atropine, and also after vagotomy in the presence of atropine, adrenaline in small doses readily counters the depressor response to 0·6 mg./kg. of himandrine. The bradycardia is only reversed for the duration of the adrenaline response after atropine and bilateral vagotomy, and thereafter remains depressed.

In the spinal cat injection of a large dose of himandrine (2·0 mg./kg.) produced no great effect on the blood pressure and only caused a mild but persistent bradycardia, of smaller magnitude than in the intact animal (see Fig. 3) — approximately 10 p.c. — at this dose level as compared with approximately

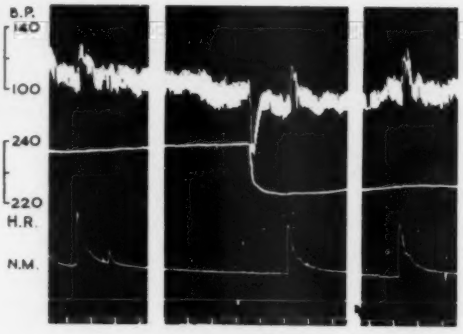

Fig. 4. The effects of himandrine on the left carotid blood pressure, heart rate, and contraction of the right nictitating membrane of the cat. The record on the left shows a control contraction of the nictitating membrane stimulated through its pre-ganglionic nerve by square wave stimuli (2 volts amplitude, 20 m. secs. duration, 20 per sec. for 10 secs.). The centre record shows the response to 0·5 mg./kg. himandrine. Two minutes after the injection the response of the nictitating membrane is unimpaired. The record on the right shows the undiminished response of the nictitating membrane eight minutes after the injection.

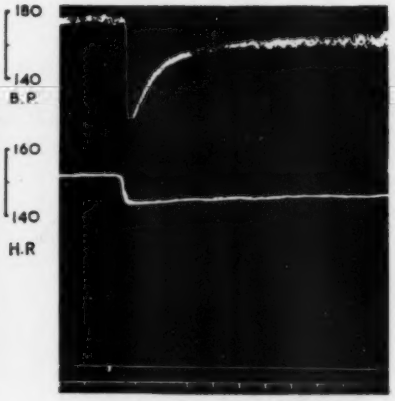

Fig. 5. The response to himandrine of the left carotid blood pressure and heart rate of the cat which have been elevated to a constant level by an infusion of adrenaline at the rate of 5 μg./kg./min., into the left saphenous vein. The record of Fig. 2 was taken from the same cat, the dose of himandrine being 2 mg./kg. in each case and the interval between the himandrine injections was 75 minutes.

47 p.c. in Fig. 2 in the intact animal receiving the same dose. These results indicate that the blood pressure fall is of central origin whereas the bradycardia appears to be partly central and partly direct in origin.

Decerebrate cats were prepared and the integrity of the medullary vasomotor centre was tested by observing a pressor response following carotid artery clamping. Injection of 2 mg./kg. himandrine produced a slight bradycardia, the heart slowing from 152 to 141 beats per minute. There was no alteration of the blood pressure in the decerebrate animal.

To test whether himandrine exerted its depressant effect by producing autonomic ganglion blockade, the right nictitating membrane of the cat was attached to a lever, writing on the kymograph. Square wave stimuli of 2 volts amplitude, 20 milliseconds duration at a rate of 20 per second were applied to the cervical sympathetic nerve trunk on the right hand side for 10 seconds, and the response recorded on the drum. After a small dose of himandrine — 0·5 mg./kg. — the usual cardiovascular response was obtained. In this experiment the cervical sympathetic trunk was stimulated at 2 minutes, 8 minutes, and 25 minutes after the injection (Fig. 4). At no time was the response of the nictitating membrane modified.

A property of several pure alkaloids derived from Veratrum species is the ability to antagonise the cardio-accelerator response to a continuous intravenous infusion of adrenaline (Krayer, 1949). Using intact cats, adrenaline was infused into the left saphenous vein at a constant rate of 5 μg./kg./min. (Fig. 5). Compare Figs. 2 and 5 which were recorded from the same animal, a time interval of 75 minutes having elapsed between the two Figures. The heart rate readily reached a plateau level and himandrine injected during the infusion caused a small fall in the heart rate, which was considerably less than when no adrenaline infusion was occurring. Six minutes after the infusion was stopped the heart rate fell from 154 to 92 beats per minute.

The depressor response to himandrine was also rapidly countered by the adrenaline infusion and the level of blood pressure stabilized within five minutes; whereas in the absence of adrenaline the same dose of 2 mg./kg. himandrine took approximately 60 minutes to stabilise in this animal.

The result of these experiments, and those reported earlier concerning the effect of single doses of adrenaline administered during the response to himandrine indicate that the response to himandrine is not mediated by blocking the normal response to adrenaline and thus, presumably, sympathetic tone at the receptor sites. Himandrine does not share the property of the Veratrum alkaloids of antagonising the cardio-accelerator response to adrenaline and it appears that the final result of administration of one drug in the presence of the other is determined by a summation of the two independent effects.

The bradycardia produced by himandrine was investigated on the isolated Langendorff heart of the cat, perfused with Ringer-Locke's solution at 37° C. aerated with 5 p.c. carbon dioxide in oxygen. When the heart was perfused

with Ringer-Locke containing 1:50,000 himandrine, complete arrest of the heart occurred in 30 seconds with a 250 p.c. increase in the coronary flow. At concentrations of 1:100,000 himandrine regularly caused a fall in the heart rate of 30 p.c., a decrease in amplitude of approximately 60 p.c., together with an increase in the coronary flow of 50 p.c. All effects were reversed by perfusion with himandrine-free Ringer-Locke. Similar effects were produced by concentrations of 1:150,000, but the intensity of each effect was diminished.

Experiments on the isolated papillary muscle preparation of Cattell and Gold (1938) were conducted to determine the effect on the contractile force of cardiac muscle. At a concentration of 1:100,000 the muscular response declined to zero in just over 5 minutes. At a concentration of 1:250,000 the amplitude of contraction declined to 50 p.c. in one hour and partially recovered when fresh buffer was introduced into the bath.

DISCUSSION.

Himandrine is a weak atropine-like agent causing relaxation of spasms induced by the choline esters acetylcholine and carbamyl choline. The greater activity against acetyl choline is very probably related to the presence of choline esterase in the preparation and the ability of this enzyme to hydrolyse acetyl choline but not carbamyl choline. However, the activity of himandrine as an atropine-like agent is weak and no further experiments were conducted on other preparations concerning this property. Similarly himandrine was found to possess only weak antihistaminic properties on the isolated intestine, having only 50 p.c. of the potency of papaverine. Further tests of antihistaminic activity were not made for this reason.

Antagonism of spasms induced by barium chloride may be criticised on the grounds of the unphysiological nature of the barium ion. Indeed, in these experiments it was difficult to obtain accurate records using barium chloride, since addition of himandrine immediately initiated violent spontaneous contractions of the muscle which, however, were removed by washing with fresh Tyrode's solution. In spite of this disadvantage, himandrine was found to have a spasmolytic potency in excess of that of papaverine by a two to threefold factor. It is generally thought that barium ions stimulate the smooth muscle of the intestine directly and not through stimulation of the release of acetyl choline from nervous elements contained within the tissue. The recent review by Vaughan Williams (1954) summarizes both sides of the controversial question of direct or indirect stimulation of intestinal muscle and indicates that the action of barium may not necessarily be solely by direct stimulation of the muscle cells. If it is, then himandrine would appear to have a more powerful musculotropic antagonising action than neurotropic.

Substances which produce the Bezold response—a fall of blood pressure together with bradycardia are always of interest to pharmacologists as possible

hypotensive agents. This response has been found after the intravenous administration of himandrine and has been shown to exist in the presence of atropine and in the vagotomised animal, and is therefore not due to stimulation of parasympathetic nerves supplying either the heart or the blood vessels. Similarly the alkaloid does not exert these actions by blocking receptors for adrenaline since the animal responds to injected adrenaline during the effects of himandrine. If the dose of himandrine is not too large, small doses of adrenaline will restore the blood pressure to its original level but only temporarily accelerate the heart to the pre-injection rate, after which it stabilises at a slower rate.

Krayer has shown that certain pure alkaloids from Veratrum species cause the Bezold reflex and also can block the cardio-accelerator action of adrenaline. The alkaloid himandrine initiates the Bezold reflex but does not share the property of pure Veratrum alkaloids in abolishing the cardio-accelerator action of adrenaline. However, himandrine does temporarily depress the heart rate when elevated by a constant infusion of adrenaline, but not to the same extent as when administered alone at the same dose level. Apparently, responses can exist to himandrine and adrenaline at the same time, and the final effect observed is due to summation of the independent effects of each drug. Since the response of the nictitating membrane to electrical stimulation remains unimpaired after the administration of himandrine, it would appear that the alkaloid does not act by blocking sympathetic ganglia.

When himandrine is administered to a spinal cat there is no alteration in the blood pressure, but a mild bradycardia is seen. Similarly in the decerebrate animal there is again no depressor response, and only a slight bradycardia. The alkaloid has been found to have an action on the isolated heart causing coronary dilatation, a diminution of the amplitude of contraction and a slowing of the rate; and on the isolated papillary muscle preparation which registers the force of contraction of a sample of heart tissue, a depressant effect is found. The results from an isolated heart need not necessarily apply *in vivo*, but the combination of results derived from the isolated heart and the papillary muscle point to the alkaloid having a direct depressant action on heart muscle which could account for at least part of the bradycardia. Since the depressor response is abolished in both the spinal and the decerebrate animal, and the heart rate is lowered, but to a lesser degree than in the intact animal, the depressor action of himandrine and the residual bradycardia are obviously mediated *via* the central nervous system.

The locus of action must be at a higher level than the medulla oblongata. It is possible on the basis of the evidence presented here, that himandrine exerts its cardiovascular actions by diminishing sympathetic tone to the heart and blood vessels and possibly the site of action is in the hypothalamic nuclei which control autonomic regulatory mechanisms. It is of interest that Plummer *et al.* (1954) have postulated this site as the target area for the action of reserpine. We have

not observed other effects such as sedation, emotional dampening, or disturbances of the heat regulating mechanism which could be explained by a hypothalamic site of action, but tentatively offer the hypothesis that himandrine exerts its cardiovascular effects by suppressing the activity of sympathetic centres of the hypothalamus.

SUMMARY.

Himandrine, a pure alkaloid derived from *Himantandra baccata*, has been studied pharmacologically.

Himandrine is not particularly toxic but in lethal doses causes convulsions followed by death.

There is no elevation or depression of the threshold to leptazol-induced convulsions in mice.

Himandrine possesses spasmolytic properties of a comparable degree to papaverine against acetyl choline, carbamyl choline, histamine and barium chloride.

Intravenous injection causes a depressor response accompanied by bradycardia, neither of which is abolished by atropine or bilateral vagotomy.

Himandrine does not block the actions of adrenaline or sympathetic ganglia in doses which are active on the cardiovascular system.

Neither does this alkaloid block the cardio-accelerator response to a constant intravenous infusion of adrenaline.

The results are discussed and a hypothesis for the mode of action of himandrine is offered.

Acknowledgment. The authors wish to thank Dr. E. Ritchie of the Department of Organic Chemistry for supplies of himandrine and are indebted to the Kahn Fund for Medical Research for the supply of experimental animals, and to the National Health and Medical Research Council of Australia for the provision of technical assistance.

REFERENCES.

Brown, R. F. C., Drummond, R., Fogerty, A. C., Hughes, the late G. K., Pinhey, J. T., Ritchie, E., and Taylor, W. C. (1956): Austral. J. Chem., 9, p. 283.

Burn, J. H. (1952): Practical Pharmacology, p. 35, Blackwell Scientific Publications.

Cattell, McK., and Gold, H. (1938): J. Pharmacol., 63, p. 116.

Cobbin, L. B., Lawson, P. T., and McFadyen, R. (1955): Austral. J. exp. Biol., 33, p. 535.

Goldberg, A. A., and Shapero, M. (1953): J. Pharm. Pharmacol., 6, p. 171.

Krayer, O., and Acheson, G. H. (1946): Physiol. Rev., 26, p. 383.

Krayer, O. (1949): J. Pharmacol., 96, p. 422.

Moran, N. C., Dresel, P. E., Perkins, M. E., and Richardson, A. P. (1954): *Ibid.*, 110, p. 415.

Plummer, A. J., Earl, A., Schneider, J. A., Grapold, J., and Barrett, W. (1954): Ann. N.Y. Acad. Sci., 59, Art. 1, p. 8.

Vaughan Williams, E. M. (1954): Pharm. Rev., 6, p. 159.

STUDIES ON INTERMEDIARY IRON METABOLISM

IX. THE EFFECTS OF PARTIAL HEPATECTOMY IN RATS

by P. S. VENKATACHALAM[1] and I. KALDOR[2]

(From the New South Wales Red Cross Blood Transfusion Service, Sydney).

(*Accepted for publication, 16th October, 1956.*)

The principal organs of iron storage are the liver, spleen and bone marrow. It has been computed that approximately one-fifth of the body iron is in the storage organs and the bulk is known to be present in the liver and spleen. The organism draws on the storage iron for the synthesis of haemoglobin. However, in a normal animal the storage iron is considered to be more or less constant. There are not many quantitative studies of changes in the storage iron compartment with particular reference to repletion following depletion of iron stores. Recently Haskins, Stevens, Finch and Finch (1952) concluded from their studies on man and dog that the iron depots once depleted by bleeding were not restored for a long time.

One of the authors has shown in an earlier experiment (Kaldor, 1955) that the removal of 30 p.c. of the storage iron in albino rats by splenectomy was made good by increased deposition of iron in the liver. It was found that at the end of the experimental period the amount of iron in the liver became equal to that held previously by liver and spleen together. The present work is an attempt to study the changes in storage iron in rats depleted of the greater part of their iron stores by the combined surgical procedures of splenectomy and partial hepatectomy.

The capacity of the liver tissue to regenerate is probably unparalleled by any other tissue or organ. This has induced both morphologists and biochemists to use partial hepatectomy as a tool in the study of tissue growth. To the authors' knowledge the technique has never been exploited for the study of iron metabolism. The rapid return of the liver to its original weight facilitated study of iron repletion at various intervals after the operation.

---

[1] This work was undertaken during tenure of a Colombo Plan Senior Medical Fellowship. Permanent address: Nutrition Research Institute, Coonoor, India.

[2] This work was undertaken during tenure of a National Health and Medical Research Council Fellowship.

METHODS AND MATERIALS.

General Plan of the Experiment.

Thirty-two male albino rats weighting between 170 and 250 gm. were divided into four groups as follows:

Group 1. Consisted of six rats to serve as controls. They were killed at the start of the experiment.

Group 2. A second control group of six rats, which were killed at the end of the experiment because all the animals used were young and actively growing.

Group 3. Consisted of ten rats on which hepatectomy and splenectomy was performed. They were sacrificed on the ninth day after operation.

Group 4. Consisted of ten rats similarly treated but sacrificed on the twentieth day after operation, at the close of the experiment.

Partial Hepatectomy and Splenectomy.

The operation of partial hepatectomy and splenectomy was performed in one stage, the former by the technique of Higgins and Anderson (1931) with slight modifications. On the day of the operation, all the animals received subcutaneously 0·5 ml. of a 1 p.c. solution of neoarsphenamine and 0·5 ml. of a solution containing 10 mg. penicillin and 5 mg. streptomycin. The control rats were similarly injected. Four rats in Group 4 died before the end of the experiment.

Diet.

All the animals received the stock diet supplied by Animal Health Products Pty., Sydney, and water *ad libitum*. The iron content of the food was found by analysis to be 20 mg. per cent.

Collection of Samples and Analytical Methods.

The blood was withdrawn by cardiac puncture and the liver removed by dissection and weighed. The haemoglobin, serum iron, iron-binding capacity, storage iron (non-haemoglobin tissue iron) values were determined using methods already described from this laboratory (Walsh, Arnold, Lancaster, Coote and Cotter, 1953; Kaldor, 1953 and 1954a). Storage iron was estimated on aliquots of the lobes of the livers removed at operation, on the spleens and on the regenerated livers removed after killing. The total serum proteins were estimated by the biuret method and the fractionation of proteins was performed by electrophoresis using the technique of Flynn and de Mayo (1951).

RESULTS.

The results are set out in Figs. 1 and 2 and Tables 1 and 2. In the construction of Table 1 and Fig. 1, the initial weights of the livers of animals in Groups 3 and 4 were calculated on the basis that the liver comprises 3·72 p.c. of the body weight as ascertained from the control animals. In a large series of male rats weighing between 200 and 300 gm., used in this laboratory, the livers were found to weigh between 3·3 and 3·8 p.c. of the body weights. Fig. 1 clearly shows the return of the weight of the liver to preoperative level in less than nine days. The regenerated liver weight expressed as a percentage of body weight was found to be 3·9 p.c. and 3·4 p.c. on the ninth and twentieth days respectively.

In the tables the tissue iron values are expressed as mg. per 100 gm. body weight to facilitate comparison of the results in the different groups. The

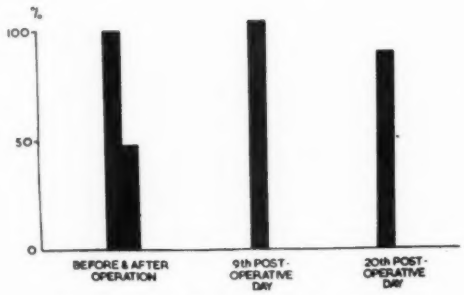

Fig. 1. Mean liver weight at four different stages in the experiment.

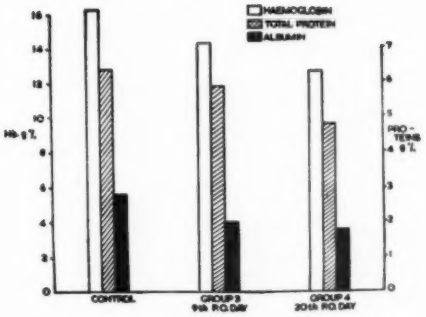

Fig. 2. Level of haemoglobin, total serum proteins and albumin at different stages.

TABLE 1.

Liver and spleen iron in the control animals.
(Mean values)

Group	No. of Rats	Body Weight (gm.)	Liver Weight (gm.)	Liver Iron (mg.) per 100 gm. Body Weight	Spleen Weight (gm.)	Spleen Iron (mg.) per 100 gm. Body Weight	Total Storage Iron (Liver +Spleen) mg. per 100 gm. Body Weight
I. Killed at start of expt.	6	223	8·4	0·27 S.D. = 0·0461	0·72	0·06 S.D. = 0·0271	0·33
II. Killed at end of expt. on 20th day	6	278	10·3	0·28 S.D. = 0·0755	0·87	0·09 S.D. = 0·0516	0·37

storage iron content of the liver and spleen of the control animals is shown in Table 1. It will be seen that the values are almost identical in spite of the fact that the animals in Group 2 grew and gained nearly 55 gm. in body weight.

The iron content of the liver and spleen in Groups 3 and 4 before the operation, immediately after the operation, and at the time of killing, is shown in Table 2. As in Fig. 1, the initial weights of the livers were calculated. The operation brought down the level of storage iron to 37 p.c. and 43 p.c. respectively in Groups 3 and 4. The results show that although the liver weight had returned to the preoperative figure by the ninth day, there was no significant addition of iron. At the end of three weeks the iron content had almost returned to the preoperative level, though less than the combined liver and spleen iron before operation.

TABLE 2.

Liver and spleen iron in the experimental animals.
(Mean values)

Group	No. of Rats	Body Weight (gm.)	Liver Weight (gm.)	Liver Iron (mg.) per 100 gm. Body Weight	Spleen Weight (gm.)	Spleen Iron (mg.) per 100 gm. Body Weight	Total Storage Iron (Liver+Spleen) (mg. per 100 gm. Body Weight)		
							Before Operation	After Operation	After Regeneration
III. On day of operation	10	189	7·0*	0·29 S.D. = 0·0402	0·78	0·06 S.D. = 0·0237	0·35	0·13	—
On 9th day after operation	10	185	7·2	0·16 S.D. = 0·0518	—	—	—	—	0·16
IV. On day of operation	6	198	7·4*	0·30 S.D. = 0·0582	0·88	0·06 S.D. = 0·0310	0·36	0·16	—
On 20th day after operation	6	273	9·2	0·27 S.D. = 0·0331	—	—	—	—	0·27

* Calculated on the basis of 3·72 p.c. body weight.

Fig. 2 shows the mean values for total plasma proteins, albumin and haemoglobin in both experimental groups and controls. There was a progressive fall in all three values. No significant changes occurred in serum iron and iron binding capacity values.

DISCUSSION.

The method commonly employed to deplete the body of iron is repeated bleeding. The withdrawal of storage iron by this indirect method is, however, a slow procedure. The operation of partial hepatectomy and splenectomy provides a quick method of removing the greater part of the storage iron. This technique of "operative iron depletion" is expected to be of value in future studies on iron metabolism.

The results of the experiment have shown clearly that during the first week after "operative iron depletion" no additional iron was deposited in the liver, while at the end of the third week the iron content of that organ had reached the original level. As a result of the increase in liver weight on the ninth day, there was a reduction in the concentration of iron per unit of liver tissue and it was assumed that the iron left in the residual lobes was uniformly distributed. To test the validity of this assumption, an experiment was conducted with another series of six rats subjected to the same operative procedure. The

animals were killed on the twelfth day after operation and five aliquots from different parts of the regenerated liver were assayed for tissue iron. The concentration of the tissue iron was approximately equal in all the aliquots.

The observations of Haskins, Stevens, Finch and Finch (1952) on the repletion of iron stores in human subjects and in dogs after repeated phlebotomy are of interest. The authors found that one year after a series of phlebotomies in a normal man the replenishment of iron stores was negligible. Fontès and Thivolle (1936) reported complete absence of storage iron in dogs with iron deficiency anaemia to whom oral iron was administered for six weeks, even though the peripheral blood picture had returned to normal. Continued administration of iron to anaemic dogs for periods varying from six to twelve months increased the storage iron to between one-third and one-half of the normal. The findings recorded in this paper indicate that the depleted iron stores in rats are replaced fairly rapidly. This difference could probably be attributed to the different method of iron depletion.

The question arises as to the source of iron for the regenerated liver. There are three possibilities: the diet, the circulating haemoglobin, and the other storage organs. This last can be excluded since the amount of iron stored in organs other than the liver and spleen is negligible. It is tempting to consider the circulating haemoglobin as the source of iron in view of the progressive decline in the haemoglobin concentration associated with the rise in liver iron, but the following observations make this unlikely.

The correlation between the fall in the haemoglobin values and the rise in the liver iron values was statistically not significant. The absence of disturbance in the peripheral blood picture and the bone marrow smears make it unlikely that iron was diverted from haemoglobin to the liver. Moreover, no abnormality of the serum iron transport mechanism was found. The fall in the haemoglobin concentration could possibly be due to haemodilution, which has been reported to occur in partially hepatectomised rats (Lowrance and Chanutin, 1942). The reduction in the total protein and albumin values could also be attributed to this haemodilution, but tissue injury and reduction of liver tissue may have played a part.

The third possibility, that the iron added to the regenerating liver tissue was derived from the diet, is being investigated in a further series of experiments. However, the diet of rats has been shown to contain a large amount of iron which is normally not absorbed, but which is readily available to anaemic animals (Kaldor, 1954b).

It is of interest that there was no iron repletion during the first week after operation, even though the liver had recovered its original weight. If the additional liver iron was eventually derived from the diet, the latent period is not unlike that reported by Hahn, Ross, Bale, Balfour and Whipple (1942) for the absorption of iron after acute haemorrhage. These workers found that in

experimental animals the rate of iron absorption from the gut remained unchanged for about a week following massive blood loss. Subsequently the amount of iron absorbed was found to increase five to fifteen times. Another possibility is that during the first week the capacity of the regenerating liver to accumulate iron may not be fully restored.

SUMMARY.

Young male albino rats were depleted of a large proportion of their storage iron by the combined operation of partial hepatectomy and splenectomy. Nine days after "operative iron depletion" the liver had regained its preoperative weight, but its storage iron content was still only one-half of the preoperative value. Twenty days after operation, however, the storage iron content of the regenerated liver had risen to the preoperative level.

The results of measurements of haemoglobin, serum protein, serum iron and iron binding capacity are given, and various possibilities for the source of storage iron in the regenerated liver are discussed.

Acknowledgments. The authors wish to acknowledge the help given and the interest taken in this work by Dr. R. J. Walsh, Director of the New South Wales Red Cross Blood Transfusion Service. Thanks are also due to Miss Marjorie Powell, for technical assistance.

REFERENCES.

Flynn, F. V., and de Mayo, P. (1951): Lancet, ii, p. 235.
Fontès, G., and Thivolle, L. (1936): Le Sang, 10, p. 144.
Hahn, P. F., Ross, J. F., Bale, W. F., Balfour, W. M., and Whipple, G. H. (1942): J. exp. Med., 75, p. 221.
Haskins, D., Stevens, A. R. (Jr.), Finch, S., and Finch, C. A. (1952): J. clin. Inves., 31, p. 543.
Higgins, G. M., and Anderson, R. M. (1931): Arch. Path., 12, p. 186.
Kaldor, I. (1953): Austral. J. exp. Biol., 31, p. 41.
Kaldor, I. (1954a): *Ibid.*, 32, p. 795.
Kaldor, I. (1954b): *Ibid.*, 32, p. 801.
Kaldor, I. (1955): *Ibid.*, 33, p. 637.
Lowrance, P., and Chanutin, A. (1942): Amer. J. Physiol., 135, p. 606.
Walsh, R. J., Arnold, Barbara J., Lancaster, H. O., Coote, Margaret A., and Cotter, Helen (1953): A Study of Haemoglobin Values in New South Wales. N.H. and M.R.C. Special Report Series No. 5, Canberra.

THE EFFECT OF URANIUM ACETATE ON SERUM LIPIDS AND ON ATHEROSCLEROSIS IN CHOLESTEROL FED RABBITS

by ALLAN J. DAY, COLIN J. SCHWARTZ,[1] GWENDOLINE K. WILKINSON and J. A. PETERS

(From the Department of Human Physiology and Pharmacology, University of Adelaide).

(*Accepted for publication, 11th October, 1956.*)

Uranium salts have been shown to elevate serum cholesterol and serum phospholipids in dogs and in rats (Heymann and Clark, 1945). These workers have attributed these changes to renal damage since bilateral nephrectomy produces a similar hyperlipemia. Uranium nitrate has also been shown to produce arterial damage. Holman (1941, 1943) demonstrated a necrotising arteritis in dogs, and these arterial changes were also shown to be dependent on the presence of kidney damage irrespective of its cause. In view of the hyperlipemia they produce, and the well recognised association of hyperlipemia and atherosclerosis, it was considered likely that kidney damage by uranium salts would also accelerate atherosclerosis. Moses and Longabough (1950) have demonstrated an increase in atherosclerosis in cholesterol fed rabbits following uranium acetate administration, but this increase occurred without any rise in serum cholesterol; in fact a marked fall occurred. In view of this apparently increased atherogenesis in the absence of increased hypercholesterolemia it was decided to reinvestigate the claim of Moses and Longabough that uranium acetate accelerates atherogenesis, and to determine whether, if such a change occurs, it parallels the serum lipid changes or occurs independently of them. In this paper, therefore, the effect of subcutaneous administration of uranium acetate on the serum cholesterol, serum phospholipids and the serum total esterified fatty acids of cholesterol fed rabbits was determined. The effect of uranium acetate on atherogenesis was also investigated and related to the lipid changes produced.

Methods.

Twenty rabbits of mongrel stock approximately six months of age (1·5-2·2 kg.) were randomised into two groups of ten rabbits in each. All animals received a daily diet consisting of 75 gm. of rabbit mash together with 1 gm. of cholesterol and 6 ml. of peanut oil.

[1] This work was aided by a grant from the National Health and Medical Research Council.

Serum cholesterol, phospholipid and total esterified fatty acids were determined prior to commencement of the cholesterol feeding and at 14 days after its commencement. At 14 days the second group of animals received subcutaneously 10 mg./kg. body weight of uranium acetate, while the first group served as a control and received an equivalent volume of 0·9 p.c. saline. Nine days after the injection (i.e. at 23 days) a further blood sample was taken and serum lipids determined as before. The blood pressures of five animals from each group were recorded 10-11 days after the uranium acetate injection by means of carotid cannulation under Nembutal anaesthesia. Subsequently all the animals were sacrificed, autopsied, their aortae removed and the atheroma present graded macroscopically. In the case of the four rabbits which died as a result of the uranium acetate treatment, four randomly selected control animals were killed at corresponding times and all eight rabbits were autopsied and the atheroma graded in their aortae.

Histopathology. Representative paraffin sections of the kidneys of both groups were prepared and stained with haematoxylin and eosin while representative frozen sections of the aortae of both groups were prepared and stained with Sudan IV.

Atheroma grading. Atheroma was graded macroscopically as described by Day and Wilkinson (1956).

Serum lipids. Serum cholesterol was determined by the method of Zlatkis, Zak and Boyle (1953), serum phospholipid by the method of Brown (1954) and total esterified fatty acids by the method of Stern and Shapiro (1953). These methods were modified slightly for use in this laboratory.

RESULTS.

Serum lipids.

Table 1 shows the serum lipid changes which occurred during the course of the experiment. Rabbits 1-10 were control animals, while rabbits 11-20 received 10 mg./kg. of uranium acetate on the fourteenth day. The serum lipids following uranium acetate administration are shown in the 23 days column compared with saline controls at the same time. The missing values at this time are due to the prior death of some of the uranium acetate animals and the corresponding sacrifice of a random control animal as has already been outlined.

Fig. 1 shows the rise in serum cholesterol which occurs in the two groups

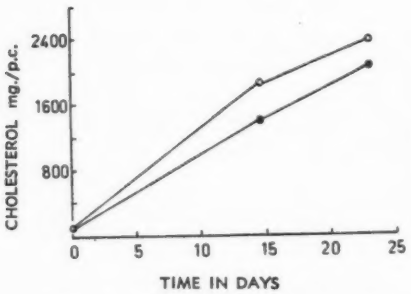

Fig. 1. The effect of uranium acetate on the rate of rise of serum cholesterol in cholesterol fed rabbits. Control group: open circles. Details in text.

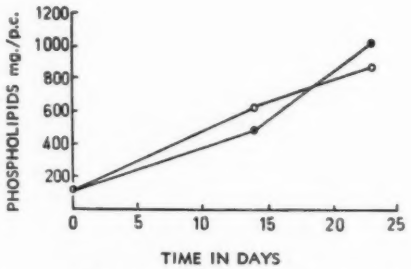

Fig. 2. The effect of uranium acetate on the rate of rise of serum phospholipids in cholesterol fed rabbits. Control group: open circles. Details in text.

TABLE 1.

Changes in serum lipids, in aortic atheroma and in blood pressure following uranium acetate injection.

Rabbit	0 Days			14 Days			23 Days			Atheroma grade	B.P.
	C*	P/L*	F/A*	C	P/L	F/A	C	P/L	F/A		
1	93	136	250	1215	474	530	1740	780	1190	I	135/125
2	96	137	188	2110	825	1170	2650	1060	1350	II	150/130
3	196	240	439	2075	760	1010	—	—	—	0	—
4	85	108	182	1950	645	960	2580	910	1400	II	145/130
5	105	142	247	1765	539	860	—	—	—	I	—
6	87	110	231	1960	637	1130	2500	880	1140	I	75/65
7	105	144	240	1960	605	710	2360	820	1000	I	—
8	96	129	203	2690	1373	1340	—	—	—	I	—
9	99	134	268	1800	555	970	2490	820	980	II	150/125
10	103	131	206	1730	605	730	—	—	—	I	—
11	99	140	250	445	211	360	1050	720	1320	0	120/90
12	78	75	167	2500	760	1070	2910	1380	2380	I	130/110
13	131	149	310	1625	539	830	2520	930	1290	I	—
14	134	110	192	1165	474	570	1570	950	1885	I	150/130
15	104	120	192	1535	515	840	—	—	—	0	—
16	132	162	270	1530	498	720	2710	1110	1650	II	100/80
17	110	130	226	1825	629	690	—	—	—	0	—
18	73	69	89	1140	368	550	1690	1040	1500	I	150/125
19	111	129	239	1975	523	1040	—	—	—	I	—
20	91	68	219	1560	508	640	—	—	—	I	—

*C = Serum cholesterol.
P/L = Serum phospholipid.
F/A = Serum total esterified fatty acids.

over the period of the experiment. It can be seen that following the administration of uranium acetate the cholesterol continues to rise in both groups at approximately the same rate. When the rise in serum cholesterol of only the six rabbits in each group surviving at 23 days is considered, a mean rise of 554 mg./p.c. occurs over the 9-day period in the control group, whereas in the uranium acetate group for the same period a mean rise of 674 mg./p.c. occurs. Statistical analysis shows that the uranium acetate has not significantly altered the rate of rise of serum cholesterol.

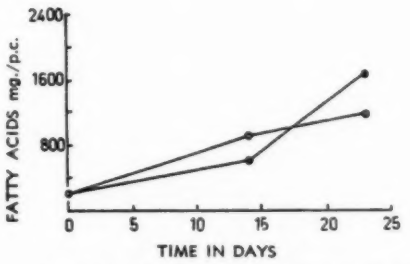

Fig. 3. The effect of uranium acetate on the rate of rise of serum total fatty acids in cholesterol fed rabbits. Control group: open circles. Details in text.

Fig. 2 shows the corresponding changes in the serum phospholipids. It can be seen from this figure that uranium acetate has greatly increased the rate of rise

of phospholipid as compared with that in the control group. Thus where only the six animals surviving in each group at 23 days are considered the mean rise in the control group over the last 9-day period is 255 mg./p.c. whereas in the uranium acetate group the mean rise is 547 mg./p.c. Statistical analysis demonstrated that uranium acetate produced a highly significant change in the rate of rise of serum phospholipids the magnitude of which was not related to the serum levels at 14 days.

Fig. 3 shows the changes in serum total fatty acid levels which occur over the period of the experiment. It can be seen that following administration of uranium acetate the rise in fatty acid level is greatly accelerated. Thus again only considering the six surviving animals in each group a mean rise of 265 mg./p.c. occurred in the control group following treatment while in the uranium acetate group a mean rise of 987 mg./p.c. occurred over the same period. The difference in the rate of rise of serum fatty acids due to uranium acetate is highly significant statistically.

Atheroma.

Table 1 also shows the macroscopic grading of atheroma in each group. Uranium acetate has produced no appreciable increase in atheroma. It is significant to note that only one animal surviving to 23 days had no atheroma and this animal showed relatively little cholesterol increase during the course of the experiment. The frozen sections prepared confirmed the fact that the lesions graded were in fact atheromatous lesions.

Blood pressure.

Table 1 shows also the blood pressures of the rabbits following treatment. The mean value in the control group was 131/115 and in the uranium acetate group 130/107. Thus uranium acetate did not produce hypertension.

Gross and microscopic pathology.

Autopsies were performed on all animals. In both the controls and uranium acetate animals, the livers were pale, abnormally friable and demonstrated the characteristics of fatty infiltration. The kidneys of the control group were macroscopically of normal appearance, while in the uranium acetate group, they were markedly pale, and the capsule tense. In this latter group, additional abnormal macroscopic findings were, an overall increase in the serous fluids, pleural, pericardial and peritoneal, and a diffuse haemorrhagic mottling on the lung surfaces.

That uranium acetate has a nephrotoxic action affecting the renal tubules of the rabbits, in a dosage of 10 mg./kg. body weight, was demonstrated. Sections of kidney from both control and uranium acetate animals were examined as paraffin sections, stained with haematoxylin and eosin. Following uranium acetate injection, there was marked hyaline necrosis of the cells of the renal tubules, which were themselves filled in many instances with an amorphous

hyaline material. A similar hyaline change was found in a few glomeruli within Bowman's capsule, but outside the glomerular tufts. The tubular necrosis was far in excess of that found normally as the result of early and rapid post-mortem autolysis in the kidneys. A moderate degree of chronic pyelonephritis was also observed in the sections of both groups.

DISCUSSION.

In this paper it has been shown that in cholesterol fed rabbits uranium acetate causes a rise in serum phospholipid and in serum fatty acids, but no appreciable rise in serum cholesterol. In a similar investigation of heavy metal intoxication Heymann and Clark (1945) demonstrated that uranium produced an elevation in phospholipid, cholesterol and total lipids in dogs and in rats. Further, their work suggested that the kidney normally aided lipid disposal and that their effects were due to the kidney damage produced. They were unable, however, to produce similar changes in rabbits. The different responses of our rabbits to the ones described by Heymann and Clark were almost certainly due to dietary differences. In normally fed rabbits lipid turnover is at a minimum, but in the cholesterol fed rabbit it is greatly accelerated. It is probable, therefore, that if the kidney plays a role in lipid disposal (as was suggested by the work of Heymann and Clark) this role, although not evident in the normally fed rabbit, would be manifested in the cholesterol fed rabbit. This contention is supported by this present work. It is probable, therefore, that the lipid increases obtained in our experiments were due to the kidney damage by the uranium acetate and consequent removal of the renal influence on lipid turnover. One further point needs to be considered. It is possible that the uranium acetate produced its lipemic effects by reducing the dietary intake. The uranium acetate animals were markedly anorexic following the injection, only consuming about one-third of the diet provided. The hyperlipemia may have been due to mobilisation of lipids during this semi-starvation. However, reduction of the dietary intake of a small group of animals to an equivalent level did not cause any appreciable change in blood lipids over the same time interval, and therefore it is doubtful whether starvation effects are responsible for the lipid changes.

The failure to support the work of Moses and Longabough (1950) in increasing atheroma by uranium acetate cannot be easily explained. Uranium acetate does cause arterial changes secondary to renal damage as was demonstrated by Holman (1941, 1943), but these changes were in the nature of a necrotising arteritis rather than atheromatous. They were accentuated in the presence of a lipid dietary factor (Holman, 1946), but at no time were they considered to be atheromatous. The occurrence of the increased atheroma described by Moses and Longabough is surprising also in view of the reduction in serum cholesterol they recorded in the uranium acetate group. Accelerated atherogenesis would be expected if the cholesterol level had been increased. In

the absence of this rise, however, a primary lipid cause for the increased atherogenesis would not be tenable and if one accepts their findings, one must accept an effect of uranium acetate either *via* the kidney or directly on the artery in either case producing atherosclerosis, i.e. a cause analogous to that producing the necrotising arteritis described by Holman. The fact that Moses and Longabough recorded substantial increases in the blood urea nitrogen may suggest that an associated hypertension was a factor in their series. Hypertension has been shown to accelerate atherogenesis in cholesterol fed rabbits (Heptinstall and Bronte Stewart, 1954). In our uranium acetate rabbits, however, there was no hypertension so that this factor was not involved in our work. The fact that we used relatively large doses of uranium acetate (10 mg./kg.) and that 4 of the 10 animals died early in this group would suggest that sufficient uranium acetate was used. Further, definite histopathological changes in the kidney were demonstrated. Whatever the cause of the discrepancy between our work and that of Moses and Longabough we certainly could not produce, under our experimental conditions, any increase in atheroma by the administration of uranium acetate.

SUMMARY.

Uranium acetate administration in cholesterol fed rabbits produces a highly significant increase in the rate of rise of serum phospholipid and serum fatty acids, but no significant change in the rate of rise of serum cholesterol.

The uranium acetate administration did not cause any increase in the atherosclerosis resulting from the cholesterol feeding.

Acknowledgments. We are indebted to Miss E. Wadlow of the Commonwealth Antioxidant Research Project for the preparation and staining of the histopathological material and to Mr. G. N. Wilkinson of the Division of Mathematical Statistics, C.S.I.R.O., for the statistical analysis of the experimental data.

REFERENCES.

Brown, W. D. (1954): Austral. J. exp. Biol., 32, p. 677.
Day, A. J., and Wilkinson, G. K. (1956): *Ibid.*, 34, p. 423.
Heptinstall, R. H., and Bronte Stewart, B. (1954): J. Path. Bact., 68, p. 395.
Heymann, W., and Clark, E. C. (1945): Amer. J. Diseases Children, 70, p. 74.
Holman, R. L. (1941): Amer. J. Path., 17, p. 359.
Holman, R. L. (1943): *Ibid.*, 19, p. 147.
Holman, R. L. (1946): Proc. Soc. exp. Biol. Med., 63, p. 87.
Moses, C., and Longabough, G. M. (1950): *Ibid.*, 74, p. 92.
Stern, I., and Shapiro, B. (1953): J. clin. Path., 6, p. 158.
Zlatkis, A., Zak, B., and Boyle, A. J. (1953): J. lab. clin. Med., 41, p. 486.

GLUCURONURIA IN A HERBIVOROUS MARSUPIAL *TRICHOSURUS VULPECULA*

by N. T. HINKS and A. BOLLIGER

(From the Gordon Craig Research Laboratory, Department of Surgery, University of Sydney).

(*Accepted for publication, 1st October, 1956.*)

The urine of *Trichosurus vulpecula*, the common brush-tail phalanger or opossum, exhibits reducing properties owing to the presence of glucuronic acid (Hinks, 1956). This observation led to an investigation on glucuronic acid in the body fluids of *T. vulpecula*, under various conditions.

The occurrence in urine of D-glucuronic acid, its γ-lactone glucuronolactone and certain glucuronides which also directly reduce alkaline sugar reagents is called glucuronuria. D-glucuronic acid is referred to as glucuronic acid.

METHODS.

Bial's test as modified by Lasker (1950) and Tollens' test according to Hawk, Oser and Summerson (1947) were used to detect glucuronic acid, and its presence and that of carbohydrates were confirmed by paper chromatography. The chromatograms were developed in butanol : acetic acid : water or aqueous phenol and visualised with aniline hydrogen phthalate or *p*-phenylenediamine. Glucuronic acid and glucuronolactone may occur together in solution and two spots appear on the chromatogram. Glucuronic acid has an R_f of 0·12 and glucuronolactone 0·33 if developed in butanol : acetic acid : water. For hydrolysing urines 5 p.c. sulphuric acid was used.

Reducing substances were determined by the methods of Somogyi (1926, 1945). Glucuronic acid was determined by the naphthoresorcinol method (Bray, Humphris, Thorpe, White and Wood, 1952), and the orcinol method (McRary and Slattery, 1945). The two methods were compared and sometimes were found to be interchangeable in the absence of pentoses. Orcinol yields a considerably cheaper and more stable reagent which does not require ageing and which after a short period of heating gives a stable green colour. In contrast the naphthoresorcinol reagent is less stable, more expensive, and has to be aged before use. The bluish grey precipitate formed when it is heated in acid solution with glucuronic acid dissolves in butanol to give a fluorescent blue solution and the pinkish grey precipitate formed with arabinose or glucose gives a pink solution with butanol. Although the colour is unstable the reaction is more specific than the orcinol reaction which does not distinguish between pentoses and glucuronic acid. We could not confirm the claims by Jarrige (1950) that the use of copper chloride in place of iron salts materially improves the usefulness of the orcinol method.

In general the phalangers were kept on a diet of vegetables, bread and fruit. Some had received in the past a day's ration of Moreton Bay fig leaves (*Ficus macrophylla*) at fortnightly intervals. The vegetable diet will be referred to as the "normal" diet.

Glucuronolactone was administered intravenously in the ear vein or orally by intubation using a freshly prepared 10 p.c. aqueous solution. The animals were kept in metabolism cages for the collection of 24-hour urine specimens. Blood was obtained from ear veins or, when large quantities were required, by heart puncture.

RESULTS.

Urine: The urine of *T. vulpecula* was always found to be laevorotatory ($[\alpha]_D - 0{\cdot}35$ to $0{\cdot}55$ in a ldm. tube). It reduced Benedict's reagent, but an osazone could not be prepared. It reacted promptly with Bial's and Tollens' reagent, but pentose could not be demonstrated by Tauber's test (1937). These observations suggested the presence of glucuronic acid or a labile glucuronide.

The presence of glucuronic acid was confirmed by paper chromatography and a spot corresponding to that of this sugar acid was always obtained on chromatograms even from the urine of phalangers which for years had been kept on a diet of vegetables and bread. Usually only glucuronic acid was detected, but in the fasting animal a spot for glucose could frequently be seen. In the fasting animal the concentration of reducing substances, i.e. glucuronic acid and glucose, often fell below the level of sensitivity of Benedict's reaction, thus erroneously indicating the absence of reducing substances (Adams and Bolliger, 1954). In addition to glucuronic acid glucuronolactone appeared on the chromatogram after the urine had been hydrolysed with mineral acid.

TABLE 1.

Twenty-four hour excretion of glucuronic acid determined by the orcinol and naphthoresorcinol method and of reducing substances determined before and after hydrolysis.

Sex	Number of 24-hour Specimens		Glucuronic Acid* mg. per 24-hours		Reducing Substances* mg. per 24-hours	
			Orcinol Method	Naphtho-resorcinol Method	Before H	After H
M.	9	Average	122	119	104	191
		Range	46-197	36-188	41-139	75-264
F.	5	Average	77	64	72	133
		Range	44-132	40-85	47-111	62-237
M.	4	Average	99	89	90	105
		Range	66-166	36-134	56-144	57-175
F.	4	Average	166	112	106	178
		Range	55-197	55-169	45-198	70-273

* Expressed as Glucuronic Acid.
H = Hydrolysis.
The animals were usually fed on a mainly "non-glucuronogenic" diet of vegetables and bread *ad lib.*

Using the quantitative method of Bray *et al.* (1952) in which presumably all glucuronides are hydrolysed, 36 to 198 mg. of total glucuronic acid were excreted in 24 hours by the common phalanger on a vegetable and bread diet (Table 1). On an average comparable results were obtained with the orcinol method (44-197 mg.), but in a few individual determinations differences of as much as 50 p.c. were encountered between the two methods. Expressed as glucuronic acid the results obtained by measuring the direct reducing power of phalanger urine (41 to 198 mg. in 24 hours) differed on an average only slightly from those obtained by the orcinol and naphthoresorcinol methods. This suggested that the bulk of the reducing compounds present must be glucuronic acid or a labile glucuronide which yields glucuronic acid in weakly alkaline solutions such as Benedict's or Somogyi's reagent. However, after hydrolysis with mineral acid the results obtained by Somogyi's method exceeded those of the naphthoresorcinol, orcinol or direct reduction procedure, indicating the liberation of reducing substances other than glucuronic acid. This assumption is supported by the appearance on the chromatogram of a glucose spot not present before hydrolysis.

On changing the diet from vegetables and bread to Moreton Bay fig leaves

TABLE 2.

The influence of diet and of borneol administration on the excretion of glucuronic acid and substances reducing before and after hydrolysis. Borneol was administered to fasting phalangers.

Animal	Diet	Glucuronic Acid* mg. per 24-hour		Reducing Substances* mg. per 24-hour	
		Orcinol Method	Naphtho-resorcinol Method	Before H	After H
1028	Vegetable—Average†	122	119	104	191
1028	Fig leaves‡—1st day	129	67	271	734
	Fif leaves‡—2nd day	77	82	217	292
	Fig leaves‡—3rd day	73	75	54	89
	Fig leaves‡—4th day	96	111	65	95
	Fig leaves‡—5th day	83	57	48	77
1028	Eucalyptus leaves§—1st day	671	247	289	479
	Eucalyptus leaves§—3rd day	351	158	146	218
	Eucalyptus leaves§—5th day	801	323	470	463
	Eucalyptus leaves§—7th day	601	429	343	421
	Eucalyptus leaves§—9th day	601	463	343	398
1028	0·5 gm. borneol. Orally	663		151	2610
765	1·0 gm. borneol. Orally	917		248	1270

* Expressed as glucuronic acid.
† Average excretion of 9 24-hour specimens.
‡ *Ficus macrophylla.*
§ *Eucalyptus acaciaeformis.*
H = Hydrolysis.

the 24 hours' excretion of substances which reduce before and after hydrolysis increased markedly on the first, and to a lesser degree, on the second day. Glucuronic acid excretion was unaltered (Table 2). On the third and subsequent days reducing substances in the urine dropped to the limits observed after a vegetable diet and remained within these limits for a period of 2 weeks during which the animal was fed exclusively on Moreton Bay fig leaves.

On feeding a species of eucalyptus leaves (*Eucalyptus acaciaeformis*) which contain *d*-*a*-pinene, sesquiterpene, geranyl acetate (Baker and Smith, 1920), after the vegetable diet, the excretion of glucuronic acid and of directly and indirectly reducing substances increased sharply and was still elevated after 10 days of eucalyptus leaves diet. On an average the excretion of glucuronic acid, determined by the naphthoresorcinol method, and of directly reducing substances was three times, and that of substances reducing after hydrolysis ("indirectly reducing substances") twice as high on eucalyptus leaves as on vegetable food. In these experiments with eucalyptus leaves intake the results obtained by the orcinol and the naphthoresorcinol methods for glucuronic acid differed greatly, the findings with the naphthoresorcinol method being on an average only about half of those of the orcinol method (Table 2). After the administration of borneol the excretion of directly and particularly of "indirectly reducing substances" and of glucuronic acid increased markedly and borneol glucuronide could be isolated from the urine (Table 2).

Administration of Glucuronolactone. Even after a hundredfold concentration of normal serum and whole blood filtrates no glucuronic acid could be demonstrated in the blood by paper chromatography either before or after hydrolysis, and glucose seemed to be solely responsible for all the directly reducing material. These concentrated filtrates gave a weakly positive Tollens' reaction and on the paper chromatogram a faint spot could be seen with an R_f value of 0·84 in butanol: acetic acid : water and of 0·74 in aqueous phenol. These high R_f values exclude the presence of glucuronic acid. However, glucuronolactone as well as the glucose normally present could readily be demonstrated in the blood

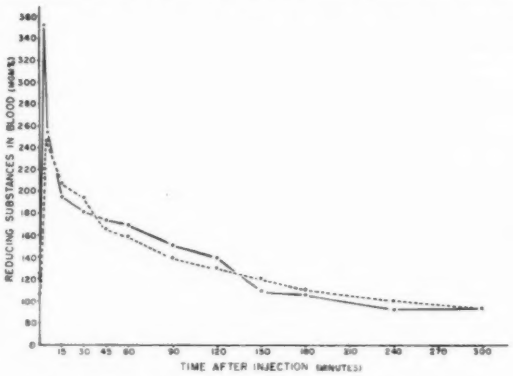

Fig. 1. Intravenous injection of glucuronolactone •——— • and of glucose • – – – – • (0·5 gm./kg. body weight). Reducing substances in blood were expressed as glucose in both experiments because the molecular weights of both substances (176 and 180 respectively) are nearly identical and the two curves are comparable. Animal No. 1155, body weight 2 kg.

stream of a phalanger to which glucuronolactone had previously been orally or intravenously administered. Fig. 1 depicts the rise of reducing substances after the intravenous injection of 1·0 gm. (0·5 gm./kg. body weight) of glucuronolactone. This rapidly disappeared from the blood stream in a manner comparable to the disappearance of an equal amount of glucose administered intravenously. Besides glucuronic acid a trace of glucuronolactone appeared in the urine after the intravenous injection of the lactone. After the injection of glucose into the same animal only glucuronic acid and no glucose was found in the urine.

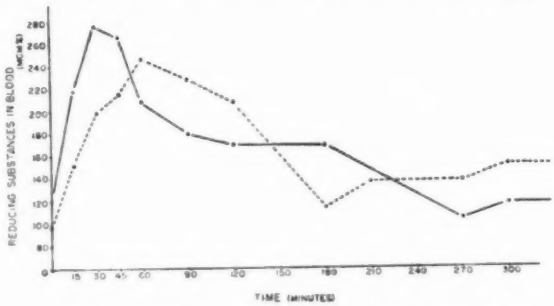

Fig. 2. Oral administration of glucuronolactone •——• and of glucose •------• (1 gm./kg. body weight). Reducing substances in blood were expressed as glucose in both experiments as in Fig. 1. Animal No. 857, body weight 1·1 kg.

Fig. 2 depicts the curve obtained by plotting the concentration of reducing substances in the peripheral blood stream against the time after the ingestion of 1 gm./kg. body weight of glucuronolactone. The characteristics of this curve are similar to the well recognised response to the oral administration of glucose.

TABLE 3.

Urinary excretion of glucuronic acid and reducing substances after the intravenous injection or oral administration of glucuronolactone.

Body Weight kg.		Glucuronic Acid* mg. per 24 hours		Reducing Substances* mg. per 24 hours	
		Orcinol Method	Naphtho-resorcinol Method	Before H	After H
2·45	480 mg. glucuronolactone—I.V. 1000 mg. glucose—I.V.	73 71	66	58 55	
2·08	650 mg. glucuronolactone—I.V. 1100 mg. glucose—I.V.	128 61	61	121 33	118
2·0	1000 mg. glucuronolactone—I.V. 1000 mg. glucose—I.V.	165 37	152	177 44	187
1·1	1100 mg. glucuronolactone—Orally 1100 mg. glucose—Orally	45 54	52 49	44 30	49 40

* Expressed as Glucuronic Acid.
H = Hydrolysis.
I.V. = Intravenous Injection.

No significant alterations in the 24-hour excretion of glucuronic acid were encountered after the oral or intravenous administration of glucuronolactone or glucose except where a large amount of glucuronolactone (500 mg./kg body weight) was injected intravenously. In this experiment directly reducing substances increased above the normal 24-hour excretion (Table 3).

DISCUSSION.

It has been demonstrated that glucuronic acid is responsible for the reducing properties of the urine of *T. vulpecula*. However, it has been often stated that free glucuronic acid does not occur in nature (Artz and Osman, 1950) and it may be possible that one is dealing with a labile glucuronide comparable to that present in the urine of rabbits fed with aniline (Smith and Williams, 1949). The existence of such a labile compound is supported by the laevorotatory properties of phalanger urine, all glucuronides being laevorotatory while the free acid is dextrorotatory. Such a hypothetical compound would also have to be sufficiently labile to be hydrolysed in the process of chromatography.

In any case the persistent glucuronuria in the phalanger, be it due to free glucuronic acid or to some directly reducing glucuronide, represents a physiological dividing line between higher mammals (eutheria) and certain marsupials (metatheria) such as *T. vulpecula* and other phalangeridae (Hinks, 1956). Higher mammals excrete stable non-reducing glycosides of glucuronic acid. Ester conjugates which reduce Benedict's solution may occur in the urine of eutheria in response to administration of compounds foreign to the organism such as benzoic acid, terpenes, etc. After the administration of certain aromatic amines free glucuronic acid or reducing glucuronides of unknown constitution may occur (Smith and Williams, 1949).

It must be emphasised that with the phalanger glucuronuria is a normal metabolic process which persisted on three different diets. On a practically non-glucuronogenic diet of vegetables, cereals and fruits directly reducing substances were always present in the urine averaging about 100 mg. of glucuronic acid in a 24-hour specimen.

After an initial shock-like increase due to a change to a diet of Moreton Bay fig leaves the excretion of reducing substances and glucuronic acid became about the same, or slightly less than as, that observed when the phalangers were fed on vegetables, etc.

A diet of eucalyptus leaves increases markedly the excretion of glucuronic acid and reducing substances for the duration of eucalyptus feeding. Therefore, it may be reasonable to assume that the glucuronuria of *T. vulpecula* is somehow connected with the ability of the phalanger to exist on its important natural source of food, the eucalypt foliage, which may contain as much as 4 gm. of essential oils, mainly terpenes, in 100 gm. of leaves. As in higher mammals the administration of a glucuronogenic terpene such as borneol leads to the forma-

tion of borneol glucuronide thus indicating the ability to deal with such substances in an eutherian manner. On the other hand it could be possible that the glucuronuria encountered in *T. vulpecula* is a general characteristic of metatheria, and in order to elucidate this point the urine of a number of marsupials including carnivores and an exclusively eucalyptus-leaf eater the native bear (*Phascolarctus cinereus*) will be investigated.

The glucuronuria of *T. vulpecula* is comparable to a benign mellituria, and may even be related to xyloketosuria in humans, where it has been suggested that xylose is formed from glucuronic acid by decarboxylation. Furthermore, it could be shown that in human pentosurics glucuronic acid may be a precursor of the xyloketose xylulose because the ingestion of glucuronolactone is followed by an increased xylulose excretion (Enklewitz and Lasker, 1935), and even normals excrete small amounts of xylulose after the ingestion of glucuronolactone (Touster, Hutcheson and Reynolds, 1954). It must be emphasized, however, that after the administration of varying amounts of glucuronolactone only glucuronic acid and occasional traces of glucose and no other reducing substances could be detected in the urine of *T. vulpecula.*

It has not been possible to demonstrate the presence of glucuronic acid in the blood of the phalanger and as in other mammals glucose is probably the only reducing saccharide present in the circulation of grown animals.

Since sodium glucuronate is to a large extent metabolically inactive and is excreted unchanged, its γ-lactone glucuronolactone is usually administered in physiological studies (Teague, 1954). In the phalanger injected or ingested glucuronolactone follows a similar pattern to that of administered glucose. It remains in the blood stream for only a short period (Fig. 1) and most of it is rapidly metabolised, and is not excreted in the urine as such or as glucuronic acid or as glucuronide except after very large intravenous doses.

In normal human subjects 46-65 p.c. of an intravenous dose of 2-3 gm. of glucuronolactone, i.e. 30-45 mg./kg. body weight, was excreted unchanged (Sudhof and Schellong, 1953). Dowben (1956) reported an excretion of 42 p.c. in one individual after the injection of 5 gm. and 26 p.c. after the ingestion of 15 gm. of glucuronolactone. In the guinea pig and in the rat it could be shown by the intraperitoneal administration of C^{14}-labelled glucuronolactone that about one half was hydrolysed to glucuronic acid and excreted in the urine, the other half being partly incorporated into glucuronides and partly metabolised to carbon dioxide (Douglas and King, 1953).

These findings suggest that in addition to the persistent glucuronuria the paradoxical retention of administered glucuronolactone is another characteristic of the glucuronic acid metabolism of *T. vulpecula.* This latter difference between marsupials and higher mammals is emphasized by the fact that relatively much larger doses of glucuronolactone were administered to the phalanger than to the eutheria examined. Also the total glucuronic acid excretion per kg. body

weight is about 3 to 7 times greater in the phalanger on a vegetable diet than in the human subject where it is approximately 7 to 14 mg. (Hawk *et al.*, 1947), and only reducing after hydrolysis.

SUMMARY.

The urine of the common brush-tail phalanger, *Trichosurus vulpecula*, a marsupial of the genus phalangeridae, always contains substances which directly reduce Benedict's solution. Glucuronic acid or a labile glucuronide was found to be the reducing substance.

When the phalanger is on a vegetable diet or on a diet of Moreton Bay fig leaves (*Ficus macrophylla*) the urinary excretion of directly reducing substances which consist of glucuronic acid is of the order of 100 mg. per 24 hours. This is trebled on a diet of eucalyptus leaves (*Eucalyptus acaciaeformis*).

Glucuronolactone administered intravenously or orally to the phalanger disappears readily from the blood-stream similar to administered glucose and neither of these substances is excreted in the urine in significant amounts, or as glucuronic acid, except after the intravenous injection of very large amounts of glucuronolactone.

Glucuronuria in *T. vulpecula*, in contrast to higher mammals, is a normal physiological phenomenon influenced quantitatively by diet.

REFERENCES.

Adams, M. D., and Bolliger, A. (1954): Austral. J. exp. Biol., 32, p. 101.
Artz, N. E., and Osman, E. M. (1950): "Biochemistry of Glucuronic Acid," Academic Press, New York.
Baker, R. T., and Smith, H. G. (1920): "A Research on the Eucalypts especially in regard to their Essential Oils," Sydney, Govt. Printer, Second Edition.
Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K., and Wood, P. B. (1952): Biochem. J., 52, p. 412.
Douglas, J. F., and King, C. G. (1953): J. biol. Chem., 203, p. 889.
Dowben, R. M. (1956): J. clin. Invest., 35, p. 277.
Enklewitz, M., and Lasker, M. (1935): J. biol. Chem., 110, p. 443.
Hawk, P. B., Oser, B. L., and Summerson, W. H. (1947): Practical Physiological Chemistry, Philadelphia, The Blakiston Co., Twelfth Edition.
Hinks, N. T. (1956): Austral. J. Sci., 18, p. 198.
Jarrige, P. (1950): Soc. Chim., biol., Bulletin, 32, p. 1031.
Lasker, Margaret (1950): Amer. J. clin. Path., 20, p. 485.
McRary, W. L., and Slattery, M. C. (1945): Arch. Biochem., 6, p. 151.
Smith, J. N., and Williams, R. T. (1949): Biochem. J., 44, p. 242, 250.
Somogyi, M. (1926): J. biol. Chem., 70, p. 599.
Somogyi, M. (1945): *Ibid.*, 160, p. 69.
Sudhof, H., and Schellong, G. (1953): Klin. Wochschr., 31, p. 64.
Tauber, H. (1937): Proc. Soc. exp. Biol. Med., 37, p. 600.
Teague, R. S. (1954): Advances in Carbohydrate Chem., 9, p. 186.
Touster, O., Hutcheson, R. M., and Reynolds, V. H. (1954): J. Amer. Chem. Soc., 76, p. 5005.

THE BIOLOGICAL ASSAY OF MELANOCYTE STIMULATING HORMONE

by BRYAN HUDSON[1] and G. A. BENTLEY[2]

(From the Baker Medical Research Institute, and Alfred Hospital Clinical Research Unit, Melbourne).

(*Accepted for publication, 1st October, 1956.*)

Various different methods have been described for the biological assay of melanocyte stimulating hormone. While melanocyte stimulation in different cold blooded vertebrates is the response common to these various methods, the criteria and methods of measurement of this response differ. Thus, response may be judged either by changes in skin colour or of individual pigment cells in living animals. Alternatively, photoelectric devices may be used to determine these changes either in living animals or in isolated pieces of skin from such animals (Landgrebe and Waring, 1950).

The purpose of this paper is to record our own investigations into the precision, sensitivity and practical usefulness of several different methods for the assay of melanocyte stimulating hormone.

Materials and Methods.

Reference Material.

For these investigations, Commonwealth Serum Laboratories Corticotrophin, Batch Nos. P.24 and P.60 were used as our reference materials. The melanocyte stimulating potency of these powders was found not to vary to any measurable extent over a period of four years.

Assays using living animals.

Animals: Both intact and hypophysectomised male *Xenopus laevis* toads were used. Their weights averaged 40 gm. and ranged between 30 and 48 gm. The animals were maintained in individual glass containers on a white background with constant overhead illumination. They were fed with raw, minced liver once weekly. Under these conditions they maintained their body weights, and when used for assay gave consistent and reproducible results. Hypophysectomy was performed according to the method described by Hogben (1923).

Procedure: The hormone, which was administered by injection into the dorsal lymph sac, was dissolved in the modified Ringer's solution described by Landgrebe and Waring (1944). The concentration was adjusted so that a dose of 0·25 ml. per 40 gm. of body weight was given.

---

[1] This investigation has been performed in partial fulfilment of the requirements of the University of Melbourne for the Degree of Doctor of Philosophy. The work has been supported, in part, by a grant from the Australian National Health and Medical Research Council.

[2] Present address: Nicholas Institute for Medical and Veterinary Research.

The index of response was the change in expansion of isolated melanocytes. The degree of expansion of melanocytes was assessed according to the scale described by Hogben and Gordon (1930). Melanocyte expansion was measured by inspection of the pigment cells in one of the webs of the hind paw, using a microscope with 20× coular, 2/3 inch objective and bright illumination.

Design: Two types of assay were used. In one, the index of response was maximal melanocyte stimulation, and in the other the degree of melanocyte stimulation ninety minutes after injection. In both assays, the level of resting melanocyte stimulation was determined before the hormone was administered, and only those toads were used whose resting melanocyte stimulation was 1·5 or less. In the former assay the degree of stimulation of thirty melanocytes was determined every 45 minutes and a curve drawn between the points (Fig. 1).

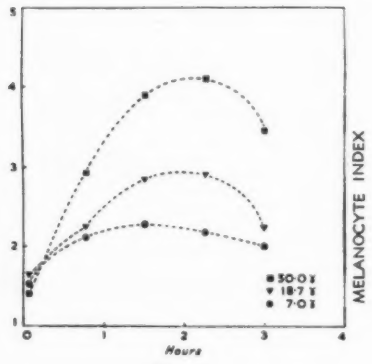

Fig. 1.

The peak of the curve was interpreted as maximal melanocyte stimulation, irrespective of the time at which it occurred. In the latter assay, the state of stimulation of fifty melanocytes at ninety minutes was determined.

Statistical Treatment: Preliminary investigations showed that an approximately linear relationship existed between log-dose and response for both types of assay. In comparing the two assays a number of observations was obtained at a variety of dose levels. This comparison showed that the method of assay in which the degree of stimulation was determined at a fixed time was more precise than when maximal melanocyte stimulation was estimated (Tables 2 and 4).

For subsequent investigations in which the sensitivity of assays using normal toads, hydrocortisone-treated and hypophysectomised toads was compared, the assays were four point in design. Sixteen toads were used with four toads at each dose level. In most assays a dose ratio of three was chosen between high and low doses of standard and unknown. Statistical analysis of these results was performed according to the recommendations laid down by Finney (1952).

Assays using isolated toad skin.

Preparation of Skin: The skin was taken either from light adapted, normal or hypophysectomised toads (*Xenopus laevis*). The animals were killed by pithing and the skin stripped from the caudal half of the body. After being washed in Ringer's solution, small portions (approximately 1·5 × 1·5 cm.) were cut from the thighs and calves. These were placed on top of small perspex holders (Fig. 2) designed especially for the purpose. The design of these holders was such that it enabled light to be directed at the surface of the skin and its reflection measured, while both surfaces of the skin were bathed in the fluid under the test. The measurement of the reflectance of light from the skin surface was made by the use of an E.E.L. reflectance meter.[3]

Use of meter: The instrument is designed to permit the use of four different colour filters and a wide range of sensitivity settings. Preliminary investigations showed that there was little, if anything, to choose between the various filters, so that in all experiments white light was used. The appropriate sensitivity settings varied from one piece of skin to the

[3] Generously lent by Thomas Optical and Scientific Company Pty. Ltd., Melbourne.

other. The sensitivity control of the instrument was adjusted so that the reading for a given piece of skin was of the order of 100, an approximate four-fifths full scale deflection. Once the level of sensitivity had been determined for a given piece of skin, it was maintained at this constant value by reference to the reflection from the matt surface of a strip of brown bakelite. Thus, with the sensitivity adjusted so that the galvanometer reading was 100 for a fresh piece of skin, the galvanometer reading for the reflection from the surface of the bakelite strip was noted. For instance, if this was found to be 20, before any further readings were taken from that piece of skin, the sensitivity of the instrument was adjusted to this value by reference to the bakelite strip.

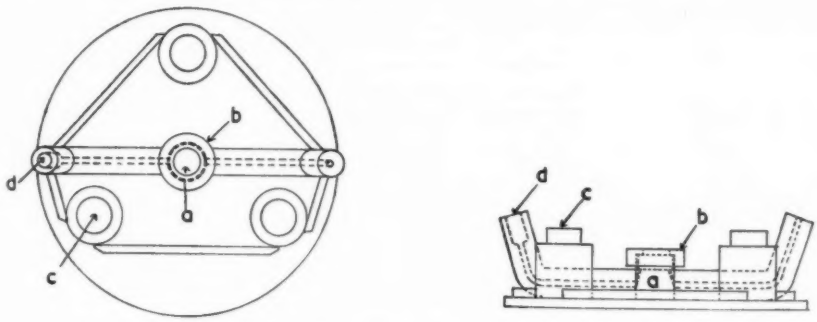

Fig. 2. Perspex holder for assay using isolated skin.
(a) Small well containing fluid under test.
(b) Ring to clip skin on to well.
(c) Supports for reflectance head.
(d) Attachment for syringe to introduce solution under test.

Assay Procedure: Three pieces of skin taken from the same animal were fixed in the frames of the perspex skin holders and allowed to stand in the modified Ringer's solution for 30 minutes, after which a suitable sensitivity level was found for each piece of skin. The Ringer's solution was then replaced by standard or test solutions. After 45 minutes the chambers and upper surfaces of the skin holders were flushed with these solutions, and after another 45 minutes the change in the skin reflectance was measured by observing the change in the galvanometer reading. The difference between the initial and final galvanometer readings was taken as the index of response. The chamber was then flushed out with Ringer's solution and the skin permitted to return to its initial level of reflectance. Once this had been reached, usually within 30-40 minutes, the procedure was repeated, and the standard and unknown solutions tested on the other pieces of skin.

Design: The assay was designed so that the response of the skin to the unknown solution lay between the response to two different concentrations of standard. Time did not usually permit the performance of an assay with two doses of both standard and unknown.

RESULTS.

Assays using living animals.

Initial investigations with such toads were directed at determining the most efficient assay procedure, later experiments at methods for increasing the sensitivity of such an assay. Over a period of seven months two methods of assay

were studied — maximal malanocyte stimulation and melanocyte stimulation at a fixed time. In Table 1 are shown the responses of normal toads to a range of doses of our reference material. The responses are the average of maximal melanocyte stimulation for 30 melanocytes for the given number of toads. Table 2 shows the regression analyses of these responses when computed against the logarithm of the dose.

TABLE 1.

Dose response results for assays using maximal melanocyte stimulation. The responses are the mean responses for the stated number of toads when the degree of stimulation of 30 melanocytes was estimated for each toad.

Dose μg./ml.	Maximal melanocyte stimulation (30 melanocytes) Hogben-Gordon index	Number of Animals
7·0	65·2	12
15·0	96·6	9
18·7	90·5	12
20·0	108·7	9
30·0	108·6	9
40·0	121·3	12
50·0	125·0	9

TABLE 2.

Statistical analysis of log-dose response figures from Table 1.

Nature of variation	d.f.	Sum of squares	Mean squares	
Between doses	6	28,564·37	4,760·73	
Linear regression	1	26,170·82	26,170·82	413·18**
Deviation from linear regression	5	2,393·55	478·71	7·55**
Within doses	65	4,117·05	63·34	
Total	71	32,681·42		

Slope of regression line = 68·517 with a standard error of 3·37.

The results of observations in which the melanocyte response is measured at ninety minutes are shown in Table 3. The responses are expressed in Hogben-Gordon indices and represent the sum of fifty melanocytes, averaged for the stated number of toads. Table 4 contains the statistical analysis of these data, the response being computed against the logarithm of the dose.

The mean of these responses plotted against the logarithm of the dose and the regression lines derived from the data are shown in Fig. 3. Inspection of Tables 2 and 4 and this Figure shows that the method of estimating melanocyte response at a fixed time gives a better log-dose response line than does the method of estimating maximal melanocyte stimulation, in which, under the circumstances of these observations, there is a significant deviation from linear regression. By contrast, the results obtained from the method of estimating

melanocyte response at a fixed time (90 minutes) show no significant deviation from linear regression. Additional advantages of the latter method are that it involves fewer manipulations and takes less time.

TABLE 3.

Dose response results for assays using the estimation of the degree of stimulation of fifty melanocytes at ninety minutes.

Dose μg./ml.	Melanocyte stimulation (50 melanocytes)	Number of animals
8·0	121·2	12
13·0	139·7	12
15·0	149·5	12
20·0	158·1	12
24·0	164·5	12
30·0	176·8	12
40·0	190·3	12
50·0	203·5	12

TABLE 4.

Statistical analysis of the log-dose response figures from Table 3.

Nature of variation	d.f.	Sum of squares	Mean square	Variance ratio
Between doses	7	60,982·14	8,717·45	
Linear regression	1	60,556·42	60,556·42	1,942·78**
Deviation from linear regression	6	425·72	70·95	2·28 N.S.
Within doses	88	2,743·30	31·17	
Total	95	63,725·44		

Slope of regression line = 101·72 with a standard error of 2·31.

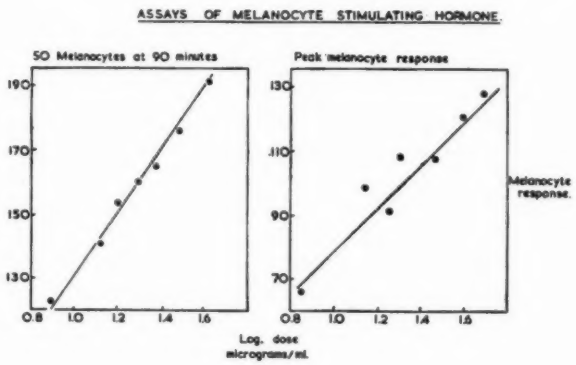

Fig. 3.

As a result of these preliminary observations a standardised assay technique was evolved. This technique consisted of the use of a self-contained assay in which two doses of unknown material were compared with two doses of standard. Four toads were used at each dose level, and, in this manner, the potency of various preparations could be compared. Such a comparison, in which the potency of LRW_1 posterior lobe powder is compared with our reference powder, is shown in Tables 5 and 6.

TABLE 5.

Assay results comparing potency of LRW_1 powder with standard reference powder.

Dose (μg./ml.)	Response: 50 melanocytes at ninety minutes			
P.60 ACTH 13·3	133·5	129·5	128·5	120·5
P.60 ACTH 40·0	175·5	174·0	188·5	172·5
LRW_1 Powder 0·2	123·5	109·0	132·5	125·0
LRW_1 Powder 0·6	158·0	172·5	163·5	161·5

TABLE 6.

Statistical analysis of results from Table 5. LRW_1 powdered estimated to be 54·8 times as potent as P.60 corticotrophin.

Source of variation	d.f.	Sum of squares	Mean square	Variance ratio
Preparations	1	370·5625	370·5625	6·78*
Regression	1	8281·0000	8281·0000	151·6**
Slope	1	68·0625	68·0625	1·25 N.S.
Between doses	3	8719·625		
Within doses	12	655·375	54·62	
Total	15	9375·00		

Although this assay technique is simple and precise, it appears to lack sensitivity. The minimum dose of our reference material to which normal toads are sensitive is of the order of 10 μg./ml. Assays of blood extracts in which there was presumed to be melanocyte stimulating activity were repeatedly negative. Investigations were made into methods that might enhance the sensitivity of assay animals, while preserving the simplicity and precision of this technique.

For this reason we tried the effects of treating toads with hydrocortisone prior to assay. It was thought that this drug might inhibit pituitary activity and thus increase sensitivity to melanocyte stimulating hormone. When toads

were given daily intraperitoneal doses of from 0·5 to 5·0 mg. of hydrocortisone acetate over periods ranging from 5 to 10 days prior to assay, there was no significant increase in their sensitivity to melanocyte stimulating hormone. Hypophysectomy, however, increases the sensitivity of toads to melanocyte stimulating hormone. In a series of three experiments, hypophysectomised toads were found to be approximately five times as sensitive to melanocyte stimulating hormone as normal, light adapted animals. Even this conspicuous increase in sensitivity was insufficient to determine the amount of hormone present in the blood of normal persons, so that for the assay of such samples the technique using isolated skin as described above was developed.

Assays using isolated skin.

Table 7 shows the responses of nine pieces of skin taken from three different toads to a ninefold dose range of P.60 corticotrophin. The figures in the Table represent differences in the galvanometer readings resulting from changes in reflectance from the skin when pale, and when darkened by hormone.

TABLE 7.

Responses of the skins from normal, light adapted toads to doses of P.60 corticotrophin.

Dose (μg./ml.)	Change in reflectance (galvanometer readings)		
	Skin 1	Skin 2	Skin 3
0·044	3·0	0·5	4·5
0·133	14·5	17·0	15·0
0·4	21·5	24·5	15·5
0·044	3·0	3·5	1·0
0·133	13·5	14·5	14·5
0·4	23·5	31·0	25·5
0·044	0·0	3·5	2·0
0·133	21·0	16·5	18·5
0·4	22·0	24·0	25·5

Regression analysis (Table 8) of these responses on the basis of log-dose response shows that a reasonably good linear relationship exists.

TABLE 8.

Regression analysis of the responses of isolated skin of normal toads.

Source of variation	d.f.	Sum of squares	Mean square	Variance ratio
Between doses	2	2105·987		
Linear regression	1	2046·634	2046·634	244·4**
Not accounted for by linear regression	1	59·353	59·353	7·09*
Within doses	24	200·99	8·375	
Total	26	2306·977		

Table 9 contains the responses from six pieces of skin from two hypophysectomised toads. Comparison of these results with those contained in Table 7 shows that such skins are more sensitive than skins from normal toads.

TABLE 9.

Responses of the skin from hypophysectomised toads to doses of P.60 *corticotrophin.*

Dose μg./ml.	Changes in reflectance (galvanometer readings)		
	Skin 1	Skin 2	Skin 3
0·02	4·0	7·5	2·5
0·06	17·5	17·0	16·5
0·18	21·0	28·0	31·0
0·02	8·5	4·5	6·5
0·06	12·0	19·0	17·5
0·18	23·5	21·5	25·5

The results of two assays using normal toad skin on the extracts of blood from a normal person and from a patient suffering from Addison's disease are shown in Tables 10a and b (Hudson and Bentley, 1957). These demonstrate the manner in which this assay procedure may be used to estimate the concentration of melanocyte stimulating hormone in unknown solutions.

TABLE 10(a)

Assays of blood extracts from a normal person (Table 10a*) and from a person suffering from Addison's disease (Table* 10b*), using the method of assay by isolated skin (non-hypophysectomised toads). Blood levels of melanocyte stimulating hormone were approximately* 1.6 *μg./*100 *ml. and* 5.4 *μg./*100 *ml. respectively.*

Dose μg./ml.	Changes in reflectance (galvanometer readings)		
	Skin 1	Skin 2	Skin 3
0·05	6·5	4·5	5·5
0·3	19·5	21·0	21·0
Blood extract	11·5	10·5	10·0

TABLE 10(b)

Dose μg./ml.	Changes in reflectance (galvanometer readings)		
	Skin 1	Skin 2	Skin 3
0·05	5·5	4·5	Skin
0·3	27·5	31·0	failed to
Blood extract	21·0	24·5	respond

DISCUSSION.

During the past five years there has been much controversy concerning the chemical and biological relationship between corticotrophin and melanocyte stimulating hormone, and about the rôle these hormones play in the pigmentary disturbances seen in patients suffering from Addison's disease. The observations reported in this paper are part of a wider investigation into this latter problem. Implicit in such a study was the need for a simple, reliable, precise and sensitive assay procedure.

At the time when this study was initiated, the most authortative account on the assay of melanocyte stimulating hormone was that given by Landgrebe and Waring (1950). They reviewed the problem with particular respect to the type of preparation to be used: *in vivo* or *in vitro* assay, the choice of animal, and the best method of determining response. These authors favoured assays using intact animals, rather than isolated preparations, and raised what appeared to be valid objections against the use of isolated skin. They claimed that isolated skin possessed two distinct deficiencies, namely, a slow response and failure of melanocytes to return to their fully contracted state. It is apparent from the observations reported in this paper that these objections cannot be sustained. We would agree that the effector speed is relatively slow, inasmuch as it takes approximately seven hours to obtain three observations from a single piece of skin. The problem of the failure of melanocytes to return to their fully contracted state does occasionally arise. In several experiments, not recorded herein, the reflectance has decreased in response to the application of hormone, and, following its removal the skin has remained dark for many hours. We have regarded this as a manifestation of a response to a supramaximal dose, and considered the failure of the skin to lighten to be due to "paralysis" of the pigment cells, although we have no direct evidence of this phenomenon. The fact that isolated skin can be used successfully for the assay of melanocyte stimulating hormone has been independently reported by Lerner and his colleagues (1954) who have used changes in the skin reflectance of *Rana pipiens* for this purpose. Frieden, Fishbein and Hisaw (1948) have also reported a method of assay using isolated frog's skin. This method relies upon the measurement of changes in the transmission of light through the skin when mounted in the cell of a photoelectric colorimeter. Using Xenopus skin and a "Spekker" colorimeter, we were unsuccessful in obtaining consistent or reproducible results.

It is apparent that the method of using isolated skin for the assay of melanocyte stimulating hormone is tedious and time-consuming when compared with *in vivo* methods. The method, however, has provided us with a distinct advantage, namely, an approximate 100-150-fold increase in sensitivity, without which the small amounts of hormone which circulate in blood could not be detected.

We have had experience with only two species: *Xenopus laevis* and the Queensland tree toad (Cheiroleptes). Species of Rana indigenous to this

country are difficult or impossible to obtain. There is no doubt that Xenopus is an extremely reliable animal and a colony of toads is readily maintained. They are easy to handle and able to lead a solitary existence in individual containers while being used for assay. Although hypophysectomy is fairly simple, the immediate mortality from this procedure is of the order of 20 p.c. We have been able to maintain a colony of hypophysectomised animals alive and well for more than sixteen months.

Landgrebe and Waring (1950) have raised theoretical objections to assays based on macroscopic colour changes, in that it was never possible to be certain whether a dark skin was due to the presence of many contracted melanocytes or a few expanded ones. It would seem that the measurement of melanocyte stimulating properties based upon the visual determinations of macroscopic colour changes could, at best, be regarded as an "all or none" procedure. Thing (1952) has shown that there is little to choose between the measurement of melanocyte stimulation by direct inspection of the pigment cells or the photoelectric measurement of colour changes in the skin. In our experience, the measurement of melanocyte stimulation by direct inspection of one of the webs of Xenopus is simple and reliable. It does not take long for an untrained person to become accustomed to assessing melanocyte response using the arbitrary scale devised by Hogben and Slome (1931).

Inspection of the dose-response figures contained in this paper leaves little doubt that, if intact animals are used, the determination of response at a fixed time is superior to the method of estimation of maximal melanocyte response. This is confirmed by the regression analysis. In their recent paper on the assay of melanocyte stimulating hormone, Ketterer and Remilton (1954) have used the same method and time relationship for the determination of response.

Early in this investigation we attempted to measure melanocyte stimulation in isolated pieces of skin by microscopic examination. We have consistently found this impossible and are of the opinion that the melanocyte density in the pieces of skin used from the thigh or calf is too high to permit adequate discrimination. Even in those pieces of skin in which there had been a conspicuous change in colour to naked eye examination, attempts at microscopic determination of melanocyte stimulation have yielded negative or inconclusive results.

In conclusion, the results that we have obtained over the past four years lead us to believe that the type of assay described, using living toads and measuring response at ninety minutes, is a simple, reliable and precise method of biological assay. It is completely satisfactory for the assay of samples which contain substantial amounts of melanocyte stimulating hormone. When unknown samples contain but small amounts of this hormone, e.g. with blood, the method is insufficiently sensitive for this purpose, and such amounts may only be detected by the use of isolated skin and a photoelectric device. This method is by no means as simple, reliable or precise, but is associated with 100-150-fold increase in sensitivity.

These results have also shown that hypophysectomy increases the sensitivity of both living animals and isolated pieces of skin to MSH. In the former, the sensitivity is increased some five times while in the latter the increase is of the order of two to threefold.

SUMMARY.

Methods for the biological assay of melanocyte stimulating hormone have been described.

A comparison has been made between assays in which maximal melanocyte stimulation and melanocyte stimulation at a fixed time (90 minutes) are used as the index of response in living toads (*Xenopus laevis*).

A comparison has also been made between assays using isolated skin from *Xenopus laevis* and assays using the living animals.

It is concluded that the technique using isolated skin enhances the sensitivity approximately 100-150-fold, but with this increased sensitivity there is a distinct loss in the precision of the assay.

The sensitivity of both living animals and isolated skin may be increased by preliminary hypophysectomy.

Acknowledgments. The authors are indebted to Professor H. Waring for his advice in the early stages of these experiments, to Mr. Kenneth Jamieson for his assistance with hypophysectomy and to Miss Diane van Assche for her technical assistance.

REFERENCES.

Finney, D. J. (1952): In "Statistical Method in Biological Assay". Charles Griffin & Co. Ltd.: London.
Frieden, E. H., Fishbein, J. W., and Hisaw, G. L. (1948): Arch. Biochem., 17, p. 187.
Hogben, L. (1923): Quart. J. exp. Physiol., 13, p. 177.
Hogben, L., and Gordon, C. (1930): J. exp. Biol., 7, p. 286.
Hogben, L., and Slome, D. (1931): Proc. Roy. Soc. Lond., B, 108, p. 10.
Hudson, B., and Bentley, G. A. (1957): In the press.
Ketterer, B., and Remilton, E. (1954): J. Endocrinol., 11, p. 7.
Landgrebe, F. W., and Waring, H. (1944): J. exp. Biol., 33, p. 1.
Landgrebe, F. W., and Waring, H. (1950): In "Hormone Assay". Ed. C. W. Emmens. New York: Academic Press.
Shizume, K., Lerner, A. B., and Fitzpatrick, T. B. (1954): Endocrinology, 54, p. 553.
Thing, E. (1952): Acta endocrinol., 10, p. 295.

RECOMBINATION BETWEEN VIRULENT AND NON-VIRULENT STRAINS OF INFLUENZA VIRUS

1. THE SIGNIFICANCE OF HETEROZYGOSIS

by PATRICIA E. LIND and F. M. BURNET[1]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(*Accepted for publication, 6th September, 1956.*)

One of the most interesting phenomena associated with the interchange of marker characters which occurs when certain pairs of influenza virus strains are grown together in the same cells is the redistribution of the virulence characters of the parent strains among the recombinant clones. A transfer of chick embryo pathogenicity (Burnet and Lind, 1952; Lind and Burnet, 1953), mouse neurotropism (Burnet and Lind, 1951a and b; Burnet and Edney, 1951; Lind and Burnet, 1954) and mouse lung pathogenicity (Perry, van den Ende and Burnet, 1954) from a strain with one of these virulence characters to a hitherto non-virulent, serologically-distinct, strain has been demonstrated a number of times. In almost all cases the acquired pathogenicity has been less than that of the donor strain. Various hypotheses have been advanced in an attempt to explain the possible mechanisms of recombination which would account for these findings (Burnet and Lind, 1954a).

In the present series of experiments a study has been made of the interchange of virulence characters between the strain NWSE which is neurotropic for mice, highly pathogenic for mouse lung and kills chick embryo when inoculated by the chorio-allantoic route, and the strain MEL which is non-neurotropic, non-pathogenic for chick embryos and which, on intranasal inoculation into mice, causes a non-fatal pulmonary consolidation involving less than half the total lung area.

In the course of this work special attention was paid to the occurrence of heterozygosis. In their recent work on recombination between the influenza strains MEL and WSN, Hirst and Gotlieb (1955), found that they were able to isolate recombinants only from heterozygous clones obtained by limit dilution titration of the harvest from the doubly infected chick embryos, and inferred that heterozygote formation was a necessary preliminary to recombinant formation. As this finding differed from our experience in the past, special attention was

[1] Supported by grants from the National Health and Medical Research Council, Canberra.

paid to heterozygosis and all the virus clones obtained from recombination experiments in this study were tested by Hirst and Gotlieb's method for the presence of serologically heterotypic virus. The results indicated that heterozygosis could be demonstrated only in a small proportion of clones, and the first of the present two papers is concerned primarily with these findings and their interpretation in the light of Hirst's contrasting results.

In the second paper the recombinant strains isolated from heterozygotes or as pure clones are described and compared, particularly in regard to virulence on intracerebral injection in mice.

MATERIALS AND METHODS.

The materials and methods described below cover both papers of this series.

Virus strains.

Neuro-WSE (NWSE). A neurotropic recombinant strain described by Lind and Burnet (1954). This strain has had 7 limit dilution allantoic passages and 2 intracerebral passages in mice since isolation.

Melbourne (MEL). A Type A strain of influenza isolated by Burnet (1935) which has had many egg and mouse passages.

Diluents.

Saline. 9 gm. NaCl made up to 1000 ml. with distilled water.

Normal Horse Serum Saline (NHSS). Saline containing 10 p.c. inactivated normal horse serum.

Ringer's Solution containing Glucose and Antibiotics (GPSR). Ringer's solution (0·85 gm. NaCl, 0·025 gm. KCl and 0·03 gm. $CaCl_2$ made up to 100 ml. with distilled water) to which glucose 0·1 p.c., penicillin (20 units/ml.) and streptomycin (70 μg./ml.) are added.

Determination of marker characteristics.

Five marker characters distinguish the strains NWSE and MEL from one another. They are determined as follows.

Aa. Serological type. This was determined by haemagglutination inhibition tests adding virus diluted to a haemagglutinin (HA) titre of 5, i.e. 5 agglutinating doses or AD, to equal volumes of 1/200 dilutions of anti-MEL and anti-WS hyper-immune rabbit sera which had been pre-treated with potassium periodate to enhance their specificity (Burnet and Lind, 1954b). Only in special circumstances was a more elaborate test used and these are mentioned in the text. All viruses of the serological character of MEL were designated A, those of WSE by a.

Cc. Conversion to indicator. When heated at 56° C. for 30 minutes NWSE is converted to the indicator state, i.e. its HA becomes susceptible to inhibition by certain soluble mucoids, in this case semi-purified meconium (Curtain, French and Pye, 1953). This character is given the symbol c. MEL virus similarly treated is not converted to the indicator state and is designated C.

Dd. Inhibition of haemagglutination by sheep mucoid. When heated at 56° C. for 30 minutes NWSE virus haemagglutinin becomes susceptible to inhibition by sheep salivary mucoid (McCrea, 1953). This character is given the symbol d. MEL virus similarly treated is not inhibited by this particular mucoid and is represented by D.

Ee. Invasiveness for the chick embryo. The virus strain NWSE has the capacity, on chorio-allantoic inoculation into the fertile egg, to kill the chick embryo within 4 days with the production of haemorrhagic encephalitis and multiple haemorrhages in the muscles and lungs. This character is designated e. Eggs similarly inoculated with MEL, on the other hand, usually survive the 4-day incubation period, or, if death occurs, no haemorrhagic lesions can be detected in the embryo. This negative character is designated E.

The following technical procedure was followed. The virus inoculum was diluted in NHSS to a HA titre of 1 (1 AD) and 0·05 ml. placed on the chorioallantois of each of four 12-day chick embryos. The eggs were incubated for 3 days, then candled to detect early deaths and all surviving eggs were incubated for a further day before being opened. The method of scoring the lesions was as follows. Death on the third day scored 2, on the fourth day 1. The degree of haemorrhagic encephalitis was graded 1-3, muscle haemorrhages 1-3, lung haemorrhages 1-3, lesions in the heart 1-2 and in the spleen 1-2 making a total possible score of 15. Scores of 2 or less were regarded as non-specific and rated E, scores of 3 or more were rated e.

Ff. Pathogenicity for the mouse lung. The symbol f was given to NWSE virus which, when diluted to a titre of 1 AD and inoculated intranasally into anaesthetized 4-week-old mice caused death of the mice with complete lung consolidation within 7 days of inoculation. MEL which, when inoculated under these conditions, failed to kill mice and produced lung lesions covering less than half the total lung area, was designated F.

Gg. Neuro-pathogenicity for mice. When inoculated intracerebrally into adult (4-week old) mice NWSE virus gives rise to neurological symptoms which frequently lead to death of the mice. This character is designated g. Its absence in the MEL strain is indicated by the symbol G.

The following method was used. The virus inoculum was diluted in NHSS to a titre of 1 AD and 0·03 ml. of this dilution inoculated intracerebrally into groups of four 4-week-old mice under light anaesthesia. The mice were examined daily for 14 days to record development of convulsions and paralysis or death. At the end of this time the surviving mice were discarded.

g^1. Neuro-pathogenicity for suckling mice. Virus was diluted in NHSS to either 10 AD or 1 AD and 0·01 ml. amounts inoculated intracerebrally into groups of six 2-day-old mice. The mice were kept under observation for 3 weeks. Cannibalism on the part of the mother frequently complicated the assessment of the average death time of certain groups. If a baby mouse were completely eaten it was not included in the score and those which were partially eaten were only included if the head remained intact and the brain could be shown, by egg inoculation, to contain virus.

Tests for the heat-stability of the virus haemagglutinin (Bb) (Burnet and Lind, 1952) were not included in this series of experiments.

In terms of the marker characteristics described above the viruses NWSE and MEL have the following formulae.

NWSE acdefg
MEL ACDEFG

Preparation of first cycle fluids in the de-embryonated egg (DEE).

The technique of preparing the DEE was adapted from that of Bernkopf (1949), and that of preparing first cycle fluids from the work of Cairns and Edney (1952). Their use in recombination experiments has already been fully described (Burnet and Lind, 1954c). The essentials of the method, as used in the present study, were to remove a circular portion of shell covering the end of the egg opposite to the air sac and through it to empty the egg of all but the chorio-allantoic membrane lining the shell and the air sac and, after thorough washing, to inoculate sufficient quantity of a mixture of the two viruses, prepared in GPSR, to ensure that a large proportion of the cells would be doubly infected with at least one particle of each virus type. The inoculated eggs were fastened to a revolving disc, the shaft of which rotated at an angle of 15° from the horizontal in a special incubator maintained at a temperature of 37° C. After incubation for one hour to allow virus absorption to occur the eggs were thoroughly washed with Ringer's solution at 37° C., refilled with 5 ml. of GPSR and returned to the incubator. The contents of the eggs were harvested 7

hours after infection. This harvest, referred to as 1st cycle fluid, usually showed a HA titre of between 20 and 100 and contained virus which must have been liberated after a single growth cycle in the cells of the egg membrane.

Determination of double neutralization.

First cycle fluids were titrated for HA titre using as diluents saline and a 1/200 dilution of periodate-treated antiserum against each of the virus strains used (Burnet and Lind, 1954b). The sum of the titres obtained in the two antisera was expressed as a percentage of the titre obtained in saline. It was found that with the controls, or with artificial mixtures of the two viruses concerned, the sum of the titres in the two antisera was 50 p.c. or more of the saline titres, so that a value of less than 50 p.c. was indicative of neutralization by both antisera.

Limit dilution (LD) titration and determination of the EID 50.

Serial $\log_{10}$ dilutions of virus were prepared in NHSS and 0·05 ml. of each of the appropriate dilutions inoculated allantoically into each of four 11- or 12-day-old chick embryos. Occasionally 0·5 $\log_{10}$ and 0·3 $\log_{10}$ dilution series were used and each inoculum injected into larger groups of from 10 to 25 eggs, but these are mentioned in the text. The eggs were incubated at 35° C. for 3 days when samples of allantoic fluid were tested for the presence of virus haemagglutinin. The 50 p.c. infectious dose (EID_{50}) was calculated by the method of Reed and Muench (1938) and throughout this paper is expressed as EID_{50} for an 0·05 ml. inoculum. Only positive fluids from eggs inoculated with doses of virus beyond the EID_{50} level were investigated for recombinant and heterozygous qualities. Such fluids are referred to as limit dilution or LD fluids.

EXPERIMENTAL.

Recombination between the influenza strains NWSE and MEL.

The two viruses NWSE (acdefg) and MEL (ACDEFG) in the form of freshly harvested allantoic fluids were diluted to HA titre 20 and mixtures prepared in the proportions NWSE : MEL, 5:1, 2:1 and 1:1. Following the technique described above the mixtures were inoculated into DEE and harvests made 7 hours after infection. Portion of each of these 1st cycle fluids was ampouled and stored in a CO_2 ice box and the remainder tested for double neutralization (see Methods). Only the harvest from the DEE inoculated with the mixture NWSE2 : MEL1 showed the double neutralization phenomenon and this particular 1st cycle fluid was used in all further work.

A preliminary infectivity titration of the 1st cycle fluid was made by allantoic inoculation of 11-day chick embryos and the EID_{50} found to be $10^{-6.7}$. Serial twofold dilutions beyond the EID_{50} level were then prepared and each inoculated allantoically into 20 eggs.

Since in earlier work on recombination between the strains MEL (ACDEFG, or, in terms of its "linkage groups", ADF-CEG) and WSE (adf-ceG), the non-mouse-neurotropic line of the WS strain, two reciprocal recombinants, M + (ADF-ceG) and WS— (adf-CEG), were isolated (Burnet and Lind, 1952), it seemed likely that neurotropic and non-neurotropic recombinants similar in other respects to M + and WS — would be expected in this experiment. Accord-

ingly, all positive fluids obtained in the above titrations were characterized by *in vitro* tests for serology and indicator status (characters Aa, Cc and Dd) which made it possible to sort them into recombinant and parent type strains prior to *in vivo* testing. In addition, each fluid was passaged allantoically at low dilution (10^{-2} or 10^{-3} according to its HA titre) in a 1/10 dilution of homologous antiserum to see if it represented a pure clone of virus or if virus of opposite serological type were also present.

Of the 145 eggs inoculated with dilutions beyond the EID_{50} level of the NWSE2:MEL1 1st cycle fluid, 24 yielded virus. Among these 24 fluids 20 could be regarded as pure clones of either parent or recombinant types of virus, i.e. were homozygous, and 4 gave evidence of containing more than one type of virus, i.e. were heterozygous. The distribution of these yields is shown in Table 1.

TABLE 1.

Characteristics of virus fluids obtained by allantoic inoculation of NWSE2 : MEL1 1st cycle fluid ($EID_{50} = 10^{-6.7}$) diluted in NHSS beyond the EID_{50} level.

Inoculum	Proportion of Positive Fluids	Characteristics of Positive Fluids	
		Homozygous	Heterozygous
$10^{-7.6}$	1/45	1 MEL	
$10^{-7.3}$	6/45	1 WS− 3 M+ 1 NWSE	1 doubly neutralized fluid (Fluid A)
$10^{-7.0}$	17/55	1 WS− 5 M+ 1 MEL 7 NWSE	1 M+ Fluid B 1 MEL Fluid C 1 WS− Fluid D

As shown in Table 1, three heterozygous fluids, B, C and D, were obtained from eggs inoculated with a $10^{-7.0}$ dilution of the 1st cycle fluid.

Fluid B, which had the *in vitro* characteristics of M +, yielded NWSE when inoculated into eggs in the presence of anti-MEL serum. It was then titrated to limit infecting dilution by the allantoic route, and of the 4 eggs inoculated with $10^{-8.0}$, the highest infecting dilution, 1 was negative, 1 contained NWSE and the other 2 contained M + indicating that NWSE was present to almost the same titre as M +.

Fluid C had the *in vitro* characteristics of MEL, but was found to contain all 4 virus types, MEL, NWSE and two recombinant types with the *in vitro* characteristics of M + and WS —. Limit dilution titration by the allantoic route yielded only MEL virus to a titre of $10^{-8.5}$ and similar titration in the presence of anti-MEL serum revealed WS — to a titre of $10^{-2.5}$. Since both NWSE and M + are pathogenic for the chick embryo when inoculated onto the chorio-

allantois (character Ee) further titrations of Fluid C, diluted in appropriate antisera, were carried out by this route. M + was found to be present to a titre of $10^{-3.3}$ and NWSE to a titre of $10^{-1.6}$. Both these figures represent minima rather than absolute values as it has frequently been observed that inoculation by the chorio-allantoic route gives consistently lower titres than those found when the same virus is titrated allantoically.

The results are summarized in Table 2.

TABLE 2.

Virus content of heterozygous fluids B and C.

Fluid	Route of Inoculation	Diluent	Virus strains isolated	EID_{50} of isolated strains (log_{10})
B (*in vitro* M+)	Allantoic	NHSS	M+	−8·0
			NWSE	Approx. −8·0
C (*in vitro* MEL)	Allantoic	NHSS	MEL	−8·5
	Allantoic	Anti-MEL serum 1/20	WS−	−2·5
	Chorio-allantoic	Anti-WS serum 1/20	M+	−3·3
	Chorio-allantoic	Anti-MEL serum 1/20	NWSE	−1·6

Fluid D, which is not included in Table 2, was a WS — strain which gave rise to NWSE on chorio-allantoic inoculation and was probably derived from a heterozygote.

The most interesting of the heterozygous fluids, Fluid A, was found in one of the eggs inoculated with the $10^{-7.3}$ dilution of the 1st cycle fluid. This fluid was unique in that its haemagglutinin was neutralized by both anti-MEL and anti-WS sera as shown in Table 3.

TABLE 3.

Serological characteristics of Fluid A compared with those of MEL and WSE fluids of similar HA titre.

Virus	HA titre in different diluents		
	Saline	Anti-MEL 1/200	Anti-WS 1/200
Fluid A	1200	160	50
MEL	1200	15	1200
WSE	1200	900	15
Mixture of equal parts of MEL and WSE	1200	500	600

As this result suggested that both MEL and WS serological types were present in Fluid A, it was inoculated allantoically into eggs at low dilution (1 AD) to-

gether with anti-MEL serum and, in another series, with anti-WS serum. In the presence of anti-MEL serum WS — was produced and in the presence of anti-WS serum MEL virus was produced. Chorio-allantoic inoculation of eggs with Fluid A mixed with anti-WS serum enabled M + to be isolated and similar inoculation of the fluid mixed with anti-MEL serum enabled NWSE to be isolated from the lungs of the dead embryos.

To determine the proportions of the 4 virus types in this fluid a series of limit dilution titrations was carried out using three different diluents, NHSS, anti-WS serum and anti-MEL serum.

The combined results of a series of allantoic titrations in which NHSS was used as diluent are shown in Table 4.

TABLE 4.

Characteristics of virus fluids obtained by allantoic inoculation of Fluid A ($EID_{50} = 10^{-8.45}$) diluted in NHSS beyond the EID_{50} level.

Inoculum	Proportion of Positive Fluids	Characteristics of positive fluids	
		Homozygous	Heterozygous
$10^{-9.0}$	8/51	6 WS− 2 MEL	
$10^{-8.7}$	6/20	1 WS− 4 MEL	1 WS−
$10^{-8.5}$	10/24	8 WS− 1 MEL	1 WS−

On the basis of the titration results it can be calculated that the EID_{50} of MEL is $10^{-8.1}$ and that of WS — $10^{-8.5}$. Limit dilution titration in anti-WS and in anti-MEL serum gave a figure of $10^{-7.8}$ for MEL and $10^{-8.1}$ for WS −. In each case the titre is slightly lower than that calculated from the titrations in NHSS and may represent a slight heterologous neutralizing capacity in the two antisera. All limit dilution fluids were tested for heterologous virus by sub-inoculation in the presence of homologous antiserum. Only 2 WS — strains showed the coexistence of virus of the other serological type. In both cases the virus was of MEL character.

Chorio-allantoic titrations were also carried out using as diluents NHSS and anti-WS serum. Anti-MEL serum was not included because previous results had suggested that NWSE was present to a higher titre than M + and that NHSS would be a suitable diluent for its isolation. In NHSS the EID_{50} of NWSE was found to be $10^{-5.5}$ and in anti-WS the titre of M + was found to be $10^{-4.1}$.

These results are summarized in Table 5.

TABLE 5.

Virus content of heterozygous Fluid A.

Route of Inoculation	Diluent	Virus strains isolated	EID_{50} of isolated strains (log_{10})
Allantoic	NHSS	MEL	−8·1
		WS−	−8·5
	Anti-WS serum 1/20	MEL	−7·8
	Anti-MEL serum 1/20	WS−	−8·1
Chorio-allantoic	NHSS	NWSE	−5·5
	Anti-WS serum 1/20	M+	−4·1

DISCUSSION.

The work of Hirst and Gotlieb (1955), as well as our own experience, indicates that heterozygous particles are frequently obtained from double infections with suitably chosen strains of influenza virus. Since the situation with influenza virus is necessarily somewhat different from other genetic phenomena to which the term heterozygosis is applied, it is necessary to indicate the way in which the term is used. We regard as heterozygous an infective unit from whose progeny two or more genetic types, not arising by mutation, can be isolated.

On almost any reasonable view of the process of virus reproduction one would expect a high yield of heterozygotes from doubly infected cells, and Hirst and Gotlieb's experience is that in their MEL/WSN system this does occur. Virtually all the recombinants they obtained were derived from limit dilution fluids which could be shown to contain particles of both serological types and hence were derived from heterozygote particles.

In the related MEL/NWSE system which we have used we have failed to note this relation between heterozygosity and recombination and in fact have had serious difficulties in understanding this failure. The other perhaps related difference between Hirst and Gotlieb's results and our own is our failure to obtain, except on excessively rare occasions, doubly neutralizable virus similar to Hirst's X_2 strains (Hirst and Gotlieb, 1953), from limit dilution fluids.

From both points of view we have paid particular attention to Fluid A. This fluid was carefully titrated for its content of haemagglutinin and of infective virus using as diluents both saline, or its equivalent, and immune serum of both the appropriate types. We may analyse the significance of these titrations by compiling Table 6, in which the haemagglutinin titres of Table 3 are taken at their face value. With this particular fluid the EID_{50} was $10^{-8.5}$ giving an EID_{50}/HA ratio of $10^{5.4}$. If we accept $10^{6.0}$ as the best ratio obtainable, this suggests that approximately one-fourth of the total particles are viable and, on this basis, the equivalent infectivity to be expected is shown in the second column of Table 6. If this is compared with the observed infectivity (3rd column)

it appears that a high proportion of the pure clones of MEL or WS serological type must have been derived from particles judged as doubly neutralizable by the haemagglutinin results.

TABLE 6.

Analysis of components of Fluid A.

Virus Type	Haemagglutinin (log_{10})	%	Equivalent Infectivity (log_{10})	Observed Infectivity (log_{10})
Total virus	3·1	(100)	8·5	8·5
MEL Serology	1·7	(4·2)	7·1	8·1
WS Serology	2·3	(17·7)	7·7	8·5
DN[1]	3·0	(78·1)	8·4	6·8[2]

[1] DN = doubly neutralizable virus.
[2] Apparent titre of demonstrably heterozygous particles.

In view of the current uncertainty about the quantitative relationship between morphological particles, haemagglutinin and infectivity (Donald and Isaacs, 1954) this conclusion must be only tentative. If all the doubly neutralized virus were non-viable, the yield of infective virus from the MEL and WS component would give EID_{50}/HA ratios of $10^{6.4}$ and $10^{6.2}$ respectively which would be unusually high, but not outside the range of ratios that have been reported. It is, therefore, a legitimate criticism that there is no real evidence that doubly neutralized virus gives rise to anything more than the small observed proportion of heterozygous clones. This, however, only pushes the difficulty back to the previous generation, because, if this view is adopted, the four types of homozygous particles present in Fluid A must all be derived from a single heterozygous particle in the primary yield from the recombination procedure.

These experiments very strongly suggest (1) that at least a proportion of doubly neutralizable particles are heterozygous; (2) that a mechanism must exist by which a large proportion of the viable progeny of heterozygous particles give rise to pure homozygous clones of one or other of the four possible combinations of "linkage groups"; and (3) that demonstrable heterozygosity is not a necessary step in the production of recombinants.

In the experience both of Hirst and Gotlieb and ourselves doubly neutralizable virus fluids always give rise to a proportion of both serological types. It is a reasonable expectation therefore that heterozygotes will be particularly prone to give a relatively high proportion of doubly neutralizable particles. To understand the results in our system of MEL/WSE and related strains it appears necessary to assume that heterozygosity is an impediment to survival so that only in exceptional circumstances do we obtain doubly neutralizable virus at limit infecting dilution. The difference between our results and those of Hirst and Gotlieb would then be simply explicable if their MEL/WSN system gave rise

to heterozygotes which were only slightly less effective in survival than homozygous forms. The fact that X_2 fluids were obtained at limit dilution (Hirst and Gotlieb, 1953) points in this direction. We believe that most of our primary recombinant particles are potentially heterozygous but for reasons not yet wholly clear, it is usual for only one of the possible genotypes to develop in the progeny. In Hirst's system the rule is for both serological types to be detectable in the progeny.

SUMMARY.

An analysis has been made of the progeny resulting from recombination between the influenza virus strains MEL and NWSE with special reference to heterozygosity.

With this system most of the recombinants obtained showed no evidence that they were derived from heterozygotes.

A limit dilution fluid showing both heterozygosity and a large proportion of doubly neutralizable virus was further analysed along the same lines. It yielded all four of the possible combinations of "linkage groups".

The results are discussed and the conclusion reached that there must be some means by which potential heterozygotes can give rise to progeny of homogeneous serological character.

REFERENCES.

Bernkopf, H. (1949): Proc. Soc. exp. Biol. Med., 72, p. 680.
Burnet, F. M. (1935): Med. J. Austral., ii, p. 651.
Burnet, F. M., and Edney, M. (1951): Austral. J. exp. Biol., 29, p. 353.
Burnet, F. M., and Lind, P. E. (1951a): J. gen. Microbiol., 5, p. 59.
Burnet, F. M., and Lind, P. E. (1951b): *Ibid.*, 5, p. 67.
Burnet, F. M., and Lind, P. E. (1952): Austral. J. exp. Biol., 30, p. 469.
Burnet, F. M., and Lind, P. E. (1954a): Nature, 173, p. 627.
Burnet, F. M., and Lind, P. E. (1954b): Austral. J. exp. Biol., 32, p. 145.
Burnet, F. M., and Lind, P. E. (1954c): *Ibid.*, 32, p. 153.
Cairns, H. J. F., and Edney, M. (1952): J. Immunol., 69, p. 155.
Curtain, C. C., French, E. L., and Pye, J. (1953): Austral. J. exp. Biol., 31, p. 349.
Donald, H. B., and Isaacs, A. (1954): J. gen. Microbiol., 10, p. 457.
Hirst, G. K., and Gotlieb, T. (1953): J. exp. Med., 98, p. 53.
Hirst, G. K., and Gotlieb, T. (1955): Virology, 1, p. 221.
Lind, P. E., and Burnet, F. M. (1953): Austral. J. exp. Biol., 31, p. 361.
Lind, P. E., and Burnet, F. M. (1954): *Ibid.*, 32, p. 437.
McCrea, J. F. (1953): Biochem. J., 55, p. 132.
Perry, B. T., van den Ende, M., and Burnet, F. M. (1954): Austral. J. exp. Biol., 32, p. 469.
Reed, L. J., and Muench, H. (1938): Amer. J. Hyg., 27, p. 493.

RECOMBINATION BETWEEN VIRULENT AND NON-VIRULENT STRAINS OF INFLUENZA VIRUS

2. THE BEHAVIOUR OF VIRULENCE MARKERS ON RECOMBINATION

by PATRICIA E. LIND and F. M. BURNET[1]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(*Accepted for publication, 6th September, 1956.*)

The experiments described in the previous paper had as their second objective a comparison of the behaviour of three virulence markers in recombinations involving, on the one hand, the strain NWSE, which is highly virulent for mice by both intracerebral and intranasal routes and produces gross haemorrhagic lesions in chick embryos, and strain MEL which has a low grade pathogenicity for the mouse lung but is quite inactive in the other two tissues.

The results to be reported are based mainly on a detailed study of the recombinants described in the preceding paper and enumerated there in Table 1. A related set of experiments is included to help elucidate the two main conclusions which have been reached. This concerns the changes in neuropathogenicity observed in strains recovered from recombination experiments. In addition to the results obtained directly a representative group of clones with reduced neuropathogenicity were subjected to a short series of brain to brain passages in mice.

Materials and Methods.

Except for the details given below the materials and methods relevant to this work have been fully described in the first paper of this series (Lind and Burnet, 1957).

Virus strain WS –. A strain obtained by recombination between the two type A strains MEL and WSE (Burnet and Lind, 1952). Fourth limit dilution passage material was used as stock.

Intracerebral passage. Six four-week-old mice were inoculated intracerebrally with 0·03 ml. of virus allantoic fluid diluted to a titre of 1 AD. Two of the mice were killed on either the 3rd or 4th day after inoculation (to allow 2 passages per week) and the remaining 4 observed daily for 14 days. The brains taken from the killed mice were ground with alundum, suspended in 1 ml. of saline per brain and centrifuged. The supernatant fluid was diluted 1/10 and 0·03 ml. inoculated intracerebrally into the next set of mice. The same dilution was also inoculated allantoically into chick embryos to check that the material was infective.

[1] Supported by grants from the National Health and Medical Research Council, Canberra.

EXPERIMENTAL.

From Table 1 of the preceding paper, it will be seen that from the NWSE2 : MEL1 1st cycle fluid used in the present work there were available presumptive pure clones of 8 + and 2 WS — recombinants. In addition there were 4 fluids which gave evidence of heterozygous origin, one frank phenotypic mixture, one M + and one MEL which on growth in anti-MEL serum gave WS fluids, and one WS — strain which on testing for pathogenicity in the chick embryo gave NWSE virus. Finally, there were 2 MEL and 8 NWSE clones of the same character as the parent strains used but which, in all probability, were the progeny of doubly infected cells. This primary classification of the fluids was based wholly on *in vitro* tests for serological character and indicator status plus growth in homologous antiserum as a test for heterozygosity.

Twelve more recombinant strains (8 M + and 4 WS —) were obtained in further limit dilution titrations of the 1st cycle fluid, by both allantoic and chorio-allantoic routes, in which the virus dilutions were prepared in either anti-MEL or anti-WS serum.

Another sample of the 1st cycle fluid was diluted in anti-WS serum and inoculated intracerebrally into adult mice. Of the 8 mice receiving the largest dose (1 AD, i.e. 1/70) 2 became ill, one on the 6th the other on the 8th day after inoculation. These mice were killed and each brain suspension, on allantoic inoculation into chick embryos, yielded M + virus.

In all 18 M + and 7 WS — clones were isolated by these procedures. These recombinant strains were given one or two further limit dilution passages in the allantoic cavity, and the LD fluids at each passage tested for all *in vitro* and *in vivo* marker characters.

All M+ strains were pathogenic for chick embryos when inoculated by the chorio-allantoic route, the lesion scores ranging from 5 to 9. Embryos inoculated with the WS — strains by the same route all showed insignificant effects except for one fluid noted above which was shown to contain NWSE.

Intranasal inoculation of mice with the standard dose (1 AD) of the recombinant viruses gave lung lesion scores, in the case of M +, close to those usually found with MEL, and, in the case of WS —, close to those of NWSE, indicating that this particular virulence marker had remained linked to those controlling the serological specificity of the recombinant strains.

Intracerebral inoculation of the same standard dose showed that the 7 WS — strains were non-neurotropic for adult mice. Of the 18 M + strains tested only 6 caused deaths, and in each case, of only 1 of the 4 mice inoculated. Many of the other mice showed signs of neurological involvement, sometimes severe, but all recovered. A number of mice was completely unaffected. It was possible to isolate M + virus from the brains of sick mice 6-7 days after inoculation

indicating that multiplication of virus had occurred. Increasing the dose of virus to 10 AD instead of the standard 1 AD gave substantially the same results as the smaller dose.

It was decided that a clearer picture might be obtained of the neurotropic qualities of the M + recombinants if they were inoculated intracerebrally into suckling mice in the first two days of life. In these tests the virus inoculum was diluted to 10 AD. When tested under these conditions all 18 of the M + strains showed a uniform neuro-pathogenicity killing all baby mice within 7 to 13 days of inoculation. M + virus could be isolated from the brains of the dead mice.

None of the 7 WS — recombinants had consistent neuro-pathogenicity for baby mice. Two of the strains, on first isolation, killed some of the mice in the inoculated groups and from the brain of one mouse in each group WS — virus was isolated. Both these mice had died 7 days after inoculation. These two strains were given two further limit dilution passages and 4-6 LD fluids tested at each passage. These LD fluids were non-pathogenic on intra-cerebral inoculation. The other 5 WS — fluids produced no symptoms on first isolation or after passage. A few of the inoculated mice died but no virus could be isolated from the brains.

Strains of the parent viruses, MEL and NWSE, reisolated from the 1st cycle fluid, were also tested fully to compare their characteristics with those of the original parent lines. They were found in all respects to be the same.

The characteristics of representative recombinant strains together with those of reisolated and original parent strains are summarized in Table 1.

As will be seen from Table 1 there are no significant differences amongst the M + strains irrespective of whether they were isolated from allantoic fluid, from embryo lung after chorio-allantoic inoculation or from mouse brain. Those isolated from heterozygous strains also behaved in the same way. Similarly all the WS — strains have the same characteristics except the two strains described above.

The age of maximum susceptibility of the baby mice to inoculation with the M + strains (which, for convenience, may be termed nM + to distinguish them from the fully neurotropic NM + previously described by Lind and Burnet, 1954) was investigated in the following experiment. nM + virus was diluted to a titre of 10 AD and 0·1 ml. amounts inoculated intracerebrally into groups of 6 mice 1, 2, 3, 4, 5, 6, 7, 14, 21 and 28 days of age. In addition, a group of mice 2 days of age was inoculated with virus diluted to 1 AD. The mice were kept under observation for 18 days. The results are shown in Table 2.

The mice showed a uniform susceptibility up to 6 days of age. Most of the 7-day-old mice survived and thereafter they appeared to lose their susceptibility almost completely. In addition, the 1 AD dose appeared to be as satisfactory as the 10 AD dose. Therefore, in all further work the 1 AD dose was used and inoculated into 2-day-old mice in 0·01 ml. amounts.

TABLE 1.

Characteristics of representative nM+ and WS− recombinant strains compared with those of the parent MEL and NWSE strains.

Virus strain	Method of isolation from 1st cycle fluid	Passage No.	Serology	Indicator Status[1]		Pathogenicity for chick embryos	Pathogenicity for mouse lung	Neuropathogenicity for adult mice	Neuropathogenicity for suckling mice
				Meconium	Sheep mucoid	Lesion score[2]	Lesion score[2]	Day of death	Day of death
nM+(1)	Allantoic titration	1st isolate	MEL	I	NI	5	0·35	7 S[3] S S	6 10M[4] 10 10
		1st LD	MEL	I	NI	5			8 8 9 9M 9 11
		2nd LD	MEL	I	NI	6	0·3		7 7 7 7 7 9
nM+(2)	Chorio-allantoic titration. Virus recovered from embryo lung	1st Isolate	MEL	I	NI		0·5	SK7M[5] SK7 11 S	
		1st LD	MEL	I	NI	8	1·2	SK6M SK6 S S	8 8M 9 9 9 9
nM+(3)	Intracerebral inoculation of adult mice	1st Isolate	MEL	I	NI		0·63	S S S –[3]	
		1st LD	MEL	I	NI			S S S –	7 7 8 9 10
nM+(4)	Chorio-allantoic titration of heterozygous Fluid A[6]. Virus recovered from embryo lung	1st Isolate	MEL	I	NI				
		1st LD	MEL	I	NI	5·3			7 8 9 10 10 10
		2nd LD	MEL	I	NI	7	0·25		9 10 10 10 10
WS–(1)	Allantoic titration	1st Isolate	WS	NI	I	0	4·1		– – – – – –
		1st LD	WS	NI	I	0		– – – –	– – – – – –
WS–(2)	Allantoic titration	1st Isolate	WS	NI	I	0	5	– – – –	7 7W[7] 11 – – –
		1st LD	WS	NI	I	0			9 – – – – –
		2nd LD	WS	NI	I	0	4·8		– – – – – –

TABLE 1—*continued.*

Virus strain	Method of isolation from 1st cycle fluid	Passage No.	Serology	Indicator Status[1]		Pathogenicity for chick embryos	Pathogenicity for mouse lung	Neuropathogenicity for adult mice	Neuropathogenicity for suckling mice
				Meconium	Sheep mucoid	Lesion score[2]	Lesion score[2]	Day of death	Day of death
WS–(3)	Allantoic titration of heterozygous Fluid A[6]	1st Isolate	WS	NI	I	0			– – – – –
		1st LD	WS	NI	I	0	4·3	– – – –	– – –
MEL parent strain			MEL	NI	NI	0	1·0	– – – –	– – – – – –
MEL	Allantoic titration		MEL	NI	NI	0	0·8	– – – –	– – – – – –
NWSE parent strain		STOCK	WS	I	I	8	5·0	6 6 6 6	
		3LD fluids from next passage						6 6 8 S 3 7 7 S 6 S S S	
NWSE	Allantoic titration		WS	I	I	8	4·8	6 6 6 6	

(1) I = indicator; NI = non indicator.
(2) For scoring convention see Methods.
(3) S = mouse sick but recovered.
– = mouse showed no symptoms throughout observation period.
(4) 10M = mouse died 10 days after inoculation; nM+ virus recovered from the brain.
(5) SK7 = mouse sick and killed 7 days after inoculation.
(6) See Lind and Burnet (1957).
(7) 7W = mouse died 7 days after inoculation; WS– virus recovered from the brain.

TABLE 2.

Neuro-pathogenicity of an nM + strain for mice of different ages.

Dilution of virus	Age of mice (days)	Day of death of mice
10 AD	1	9 9 9 9 9
	2	7 9 9 9 9
	3	7 7 7 10 11 11
	4	8 10 12 12 13 s[1]
	5	7 10 10 11
	6	8 8 8 8 8 9
	7	10 s s s s s
	14	–[2] – – – – –
	21	s – – – – –
	28	– – – – – –
1 AD	2	7 7 8 8 8 8

[1] s = mouse sick but alive 18 days after inoculation.
[2] – = mouse showed no symptoms throughout the observation period.

Recombination between nM + and WS –.

In view of the fact that the neurotropic character of NWSE virus had been transferred by recombination to the nM + strain, but with a greatly diminished virulence, further experiments were carried out to see if, by back recombination between nM + and WS —, NWSE could be obtained, and whether such NWSE strains would have the weak neurotropism of the nM + strains or the full neurotropism of the original NWSE parent virus.

In these experiments the strain of nM + chosen as parent virus was a strain showing characteristics almost identical with the strain nM + (1) in Table 1. Third limit dilution passage material of this virus strain was inoculated allantoically, at 10^{-4} dilution, into chick embryos and the harvest used to inoculate DEE. It was decided to use standard WS — in place of the WS — obtained as a recombinant in the experiments described above, to remove any likelihood that some latent neurotropic quality in the recombinant WS — might be reactivated in the recombination process and thus to ensure that, if NWSE strains were obtained, they would derive their neurotropic character only from the nM + strain.

The experiment was carried out in the usual manner, DEE being inoculated with the two viruses diluted to 20 AD and mixed in the proportions, nM +:WS —, 5:1, 1:1 and 1:5. All the 7-hour harvests showed the double neutralization phenomenon. The pooled harvests from each group were diluted in anti-MEL serum and inoculated onto the chorioallantois of the 12-day-old chick embryos. Deaths occurred among embryos receiving each of the 3 inocula, the dead embryos showing the characteristic haemorrhagic lesions associated with NWSE infection. *In vitro* tests on saline extracts of the ground-up lungs showed virus of WS serology to be present in each case. These saline extracts were diluted to the 1 AD level and inoculated intracerebrally into adult and baby mice.

Most strains were given one, and in some cases two, limit dilution passages in the allantoic cavity and the LD fluids tested for their *in vitro* characteristics, pathogenicity for the chick embryo and their neuropathogenicity for adult and baby mice. In all 17 strains of WS type virus were obtained in this way. All showed the *in vitro* characteristics of WSE, all were pathogenic for chick embryos when inoculated onto the chorio-allantois and on intracerebral inoculation into mice they gave the following results. None of the strains, when tested in adult mice, killed all 4 of the mice inoculated. Of the original isolates, i.e. the saline extracts of the infected chick lungs, 6 killed 2 mice out of the 4 inoculated, all brains showing the presence of WSE type virus, 4 killed 1 out of the 4 mice and 7 did not cause any deaths although a few of the mice in these groups showed a transient illness. First and second limit dilution passage fluids gave substantially the same results as the original isolates. Intracerebral inoculation of 2-day-old mice with virus diluted to 1 AD resulted in the death of all mice between the 5th and the 11th day after infection. Brains of mice in nearly all groups were inoculated allantoically into chick embryos and WSE type virus isolated from them.

All 7-hour harvests were diluted in anti-MEL serum and inoculated intracerebrally into adult mice. Only one WSE strain was isolated by this means and its neurotropic qualities were very similar to those of the WSE strains isolated from the chick embryo lungs.

Only one WSE strain was obtained by allantoic inoculation of the 7-hour fluids after dilution in anti-MEL serum, WS — virus tending to overgrow the recombinant WSE in all titrations of this kind. It too was similar to the strains described above.

Heterozygous strains were not sought in this experiment.

The characteristics of representative strains of this weakly neurotropic group of WSE recombinants, nWSE, are shown in Table 3.

Six strains of the reciprocal recombinant MEL were isolated. None was neuropathogenic. The characteristics of a typical MEL strain and strains of WS — and nM + reisolated from the 7-hour harvest are also included in Table 3. The only point calling for comment is the appearance of nM + reisolates which lack any significant neuropathogenicity for baby mice.

Serial mouse brain passage of weakly neuropathogenic strains.

All who have worked with neuro-recombinants have commented on the variability of expression of their neuropathogenicity. It was thought that some light might be thrown on this phenomenon by submitting representative strains of nM + and the back recombinant nWSE to mouse brain passage. This was done for 8 passages according to the plan described under Methods. Parallel passages were made with the original parent strain of NWSE and of our stock WSE strain. The results are shown in Table 4.

TABLE 3.

Characteristics of representative nWSE and MEL recombinant strains compared with those of the parent nM+ and WS– strains.

Virus strain	Method of isolation from 1st cycle fluid	Passage No.	Serology	Indicator status		Pathogenicity for chick Embryo Lesion scores	Neuro-pathogenicity for adult Mice. Day of death	Neuro-pathogenicity for suckling mice. Day of death	No. of strains showing similar reactions
				Meconium	Sheep mucoid				
nWSE(1)	Chorio-allantoic titration. Virus recovered from embryo lung	1st Isolate	WS	I	I	7	6 S – –	$8_{NW^{(1)}}$ 9 9	9
		1st LD	WS	I	I		9 S – –	5 6 6 7_{NW} 7 7	
		2nd LD	WS	I	I	8	– – – –	5 8 8 8 8 11	
nWSE(2)	Chorio-allantoic titration. Virus recovered from embryo lung	1st Isolate	WS	I	I		S – – –	3 3 3 3	
		1st LD	WS	I	I	5	9 – – –	7 7_{NW} 8 8	2
		2nd LD	WS	I	I	7	6 7 8 –	6 6 6_{NW} 7 7 7	
nWSE(3)	Chorio-allantoic titration. Virus recovered from embryo lung	1st Isolate	WS	I	I		S – – –	6 7 7_{NW} 7 7	
		1st LD	WS	I	I	6·5	– – – –	5 5 5 6 6 6	6
nWSE(4)	Intracerebral inoculation of adult mice	1st Isolate	WS	I	I	8	7 7 –	7 7 7 7_{NW} 8	
		1st LD	WS	I	I		7 10 20 –	8 8 8 8 9 9	1
nWSE(5)	Allantoic titration	1st LD	WS	I	I	6	6 8 – –	6 7 7 7 7 8	1
MEL	Allantoic titration	1st Isolate	MEL	NI	NI	0		– – – – – –	6
WS– parent strain			WS	NI	I	0		– – – – – –	
WS–	Allantoic titration	1st Isolate	WS	NI	I	0		– – – – – –	3

TABLE 3—*continued.*

Virus strain	Method of isolation from 1st cycle fluid	Passage No.	Sero-logy	Indicator status		Pathogenicity for chick Embryo Lesion scores	Neuro-patho-genicity for adult Mice. Day of death	Neuro-patho-genicity for suckling mice.	No. of strains showing similar reactions
				Mecon-ium	Sheep mucoid				
nM+ parent strain			MEL	I	NI	7	- - - -	7 7 7 8 9 11	
nM+(1)	Allantoic titration	1st Isolate	MEL	I	NI	7	- - - -	15 - - - - -	2
nM+(2)	Allantoic titration	1st Isolate	MEL	I	NI	7	6 - -	8 9 9 9 9 9	1

(1) 8_{NW} = Mouse died 8 days after inoculation; NWSE virus recovered from the brain.
For all other details see footnotes to Table 1.

TABLE 4.

Results of serial mouse brain passage of WSE, NWSE, nM+ and nWSE strains.

Inoculum	Virus Strain							
	WSE		NWSE		nM+		nWSE	
	Neuro-path. for mice Day of death	Infectivity for allantoic cavity No. positive/No. inoc.	Neuro-path. for mice Day of death	Infectivity for allantoic cavity No. positive/No. inoc.	Neuro-path. for mice Day of death	Infectivity for allantoic cavity No. positive/No. inoc.	Neuro-path. for mice Day of death	Infectivity for allantoic cavity No. positive/No. inoc.
Stock virus	– – – –		7 7 7 7		7 – – –		– – – –	
1st mouse brain passage virus	– – – –	1/3	7 7 – –	3/3	– – – –	2/3	10 – – –	3/3
2nd ,,	– – – –	0/3	7 7 7 7	1/3	10 S – –	3/3	10 S – –	3/3
3rd ,,	– – – –	0/3	7 7 7 7	3/3	9 S – –	3/3	S S – –	3/3
4th ,,			4 6 6 7	3/3	S S – –	3/3	S S S –	3/3
5th ,,			6 7 8	0/3	8 8 9 9	3/3	8 8 11 S	3/3
6th ,,			6 6 7	1/3	8 8 8 –	3/3	9 9 – –	2/3
7th ,,			7 7 7 7 7 8	2/3	7 7 8 9 S S	2/3	7 7 7 8 9 S	3/3
L.D. Fluid from allantoic titration of 7th mouse brain passage virus			6 7 7 7		6 6 8 S		12 S S S	

Two mice from each group were killed 3-4 days after inoculation and their brains used to provide the next inoculum as described in Methods. This Table records the scores obtained with the remaining mice in each group which were kept for observation. For details of scoring see footnotes to Table 1.

All strains behaved as would be expected. WSE was lost on the second passage. Original NWSE chosen in the first place for the comparative regularity of its neuro-pathogenicity gave consistent fatal infections despite the fact that there were indications of very low virus content in several brain suspensions. The primary recombinant nM + and the back recombinant nWSE both showed a definite but low and irregular neuro-pathogenicity with no clear suggestion of any increase in pathogenicity with passage. It must be concluded, therefore, that the characteristic behaviour of the neuro-recombinants obtained in the present experiment is a relatively stable character although it will be noted from Table 3 that two strains of nM + recovered from the back recombination experiment nM +/WS — had lost pathogenicity even for suckling mice.

As yet we have no clue as to the genetic factors at the basis of the differences between the extremely high and stable virulence of NWS (Burnet, 1951), the relatively high virulence of some chosen recombinants such as NWSE and the virulence of the present strains which is almost limited to suckling mice.

DISCUSSION AND SUMMARY.

In this set of experiments the behaviour of the neuro-pathogenic quality has differed somewhat from our experience when the original NWS strain was used as the parent in recombination experiments (Burnet and Edney, 1951; Burnet and Lind, 1951). In the first place the strain NWSE is not as consistent in its neuropathogenicity as the NWS strain. The strain with which the experiments were started killed 4-week-old mice with complete regularity in 6 days, but if a number of limit dilution fluids from a further limit dilution passage were tested, a proportion of less pathogenic clones was found. Amongst the nM + recombinants the neuro-pathogenicity for grown mice was low and variable. All, however, were highly lethal for suckling mice, a quality not shown by MEL and WS — strains. Back recombination with WS — and these weakly neuro-pathogenic nM + strains gave nWSE strains with the same weak pathogenicity essentially limited to baby mice.

The other two virulence markers studied showed very uniform behaviour. Virulence for the mouse lung (Ff) showed absolute linkage with serological character (Aa) and indicator status to sheep salivary mucoid (Dd) forming the linkage groups ADF and adf, while chick embryo virulence (Ee) was regularly linked to indicator status to meconium (Cc). No variation outside the range of normal expression of these characters was observed.

In summary the results of this series of experiments give a clear picture of the independent segregation of the 4 linkage groups ADF, CEG, adf and ceg, and show that, with our material, demonstrable heterozygosity is rare and not a necessary preliminary to recombination. They also provide evidence of an interesting modification, g′, of the virulence character g. Clones carrying the g′ marker are virulent, on intracerebral inoculation, for baby mice but not for adult mice.

Acknowledgment. The authors wish to acknowledge the highly competent technical assistance of Miss Lois Larkin during the course of this work.

REFERENCES.

Burnet, F. M. (1951): J. gen. Microbiol., 5, p. 46.
Burnet, F. M., and Edney, M. (1951): Austral. J. exp. Biol., 29, p. 353.
Burnet, F. M., and Lind, P. E. (1951): J. gen. Microbiol., 5, p. 67.
Burnet, F. M., and Lind, P. E. (1952): Austral. J. exp. Biol., 30, p. 469.
Lind, P. E., and Burnet, F. M. (1957): *Ibid.*, 35, p. 57.

THE EFFECT OF POTASSIUM AND ACETYLCHOLINE ON THE RESPONSE OF THE GUINEA-PIG JEJUNUM TO HISTAMINE

by M. J. RAND[1]

(From the Department of Pharmacology, University of Sydney).

(*Accepted for publication, 6th September, 1956.*)

Many observations have been made on the effect of altered potassium concentration of Ringer's solution on the activity of smooth muscle. Changes in the activity of the smooth muscle after it has been returned to normal potassium Ringer's solution have been investigated less frequently.

Using the isolated colon of the rat, Hazard and Cornec (1952) observed an increase in sensitivity to acetylcholine after exposure to an increased potassium concentration. Furthermore, Graham (1951) found that the guinea-pig ileum transferred from a potassium-low to a normal Ringer's solution had a diminished reactivity to acetylcholine, histamine and barium.

In the experiments reported here, the smooth muscle preparation has only been subjected to increased potassium concentration for a short period and then restored to normal Ringer's solution in order to obtain a temporarily increased intracellular : extracellular potassium ratio. To obtain a decreased intracellular : extracellular potassium ratio, the smooth muscle was treated with acetylcholine, which has been shown to liberate potassium from the muscle (Lembeck and Strobach, 1955; Born and Bülbring, 1956), and then restored to normal Ringer's solution. The responsiveness of the smooth muscle preparation to constant doses of histamine has been tested before and after exposure to potassium chloride and acetylcholine.

Methods.

Guinea-pig jejunum was suspended in Ringer's solution of the following composition: NaCl 8·0 gm., KCl 0·2 gm., $CaCl_2$ 0·2 gm., $MgCl_2$ 0·01 gm., NaH_2PO_4 0·05 gm., Na_2CO_3 1·0 gm., dextrose 1·0 gm., distilled water to 1 litre. The solution was aerated with oxygen containing 5 p.c. CO_2, and the temperature was maintained at 37° C. The volume of the organ bath was 10 ml.

Histamine dihydrochloride, acetylcholine chloride and potassium chloride were dissolved in Ringer's solution. Injections of histamine (0·2 - 0·5 μg.) were given into the bath at 2

[1] Drug Houses of Australia Research Fellow in Experimental Pharmacology.

minute intervals. The period of contact of histamine with the jejunum was held constant in each experiment and ranged from 10 to 15 seconds before washing out. When constant responses to the histamine injections were obtained, acetylcholine or potassium chloride was added to the bath and allowed to remain there for varying periods of time; after washing out, the histamine injections were continued. Responses were recorded on smoked paper with a lever giving a 4 to 1 magnification.

RESULTS.

The effect of increased potassium chloride on the response to histamine.

The effect of potassium chloride (1·0-4·0 mg. in contact for 15 sec. to 3 min.) on the response of the jejunum to histamine was observed in 8 experiments. In each of these experiments, the response to histamine was greater after treatment with potassium than it had been before. The means of the 3 responses to histamine before the addition of the potassium chloride to the bath and the 3 responses to histamine after washing out the potassium chloride are given in Table 1. The

TABLE 1.

Jejunum	Treatment	Dose added to bath	Time of contact (min.)	Mean of 3 responses to histamine		Percentage of control
				Before treatment mm.	After treatment mm.	
A	KCl	2 mg.	2	18·7	21·7	116
	KCl	4 mg.	2	21·7	31·0	143
B	KCl	2 mg.	3	32·3	91·3	251
C	KCl	2 mg.	1·5	24·7	26·0	105
E	KCl	4 mg.	2	11·7	12·0	103
F	KCl	1 mg.	1	9·3	12·7	136
	KCl	2 mg.	1	17·0	20·7	122
G	KCl	1 mg.	0·25	19·3	25·7	133
C	ACh	10 μg.	6	26·7	3·7	14
	ACh	1 μg.	4	25·0	15·0	60
D	ACh	1 μg.	3	7·3	6·3	86
	ACh	5 μg.	3	6·0	5·7	95
	ACh	10 μg.	4	5·7	5·3	93
	ACh	20 μg.	4	6·0	4·0	67
F	ACh	10 μg.	1	12·7	7·7	60
G	ACh	1 μg.	3	43·0	35·7	83
	ACh	5 μg.	3	22·3	16·3	73

increase in response of the jejunum to histamine after treatment with potassium chloride was only temporary. After treatment for 3 min. with 2 mg. of potassium chloride, the response to histamine was markedly potentiated, but 10 - 12 min. later the response had fallen back to its initial level (Fig. 1).

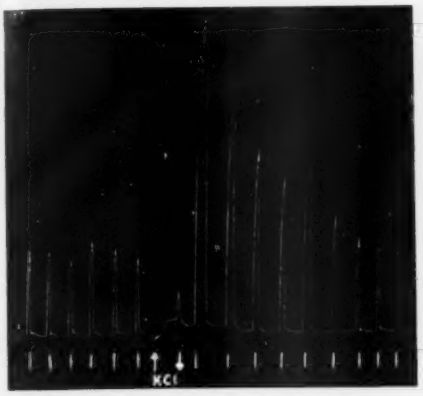

Fig. 1. Guinea-pig jejunum. The unlettered contractions are to 0·2 μg. of histamine dihydrochloride. At the sign ↑ 2 mg. of potassium chloride was added to the bath and this was washed out at ↓ after 3 minutes.

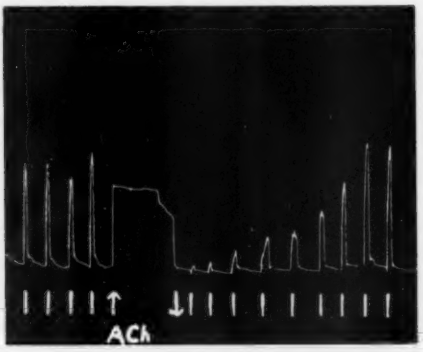

Fig. 2. The unlettered contractions are to 0·2 μg. of histamine. At ↑, 10 μg. of acetylcholine was added to the bath and washed out 6 minutes later at ↓. The lever was arrested during the ACh spasm.

The effect of acetylcholine on the response to histamine.

Treatment of the jejunum with doses of acetylcholine ranging from 1 to 10 μg. for from 1 to 6 min. reduced the subsequent responses to histamine in 9 experiments. The results of these experiments are given in Table 1, and one experiment is illustrated in Figure 2.

DISCUSSION.

On the basis of these experiments, the hypothesis is advanced that the responsiveness of guinea-pig jejunum is governed in part by the ratio of intracellular : extracellular potassium. Treatment of the jejunum with high potassium Ringer's solution followed by transfer to normal Ringer's solution would result in a temporary increase in this ratio, and there is increased responsiveness. Prolonged treatment with acetylcholine followed by transfer to fresh Ringer's solution would result in a decrease in the intracellular : extracellular potassium ratio, and produces decreased sensitivity of the jejunum.

Fenn (1940) has proposed a similar explanation for the decrease in developed tension in rhythmically stimulated skeletal muscle, from which there is a progressive loss of potassium.

SUMMARY.

The responsiveness of the guinea-pig isolated jejunum to histamine is increased after treatment with potassium chloride (150 to 300 p.c. of the concentra-

tion in normal Ringer's solution), and decreased by prolonged treatment (1 to 6 minutes) with doses of acetylcholine which produce contraction.

REFERENCES.

Born, G. U. R., and Bülbring, E. (1956): J. Physiol., 131, p. 690.
Fenn, W. O. (1940): Physiol. Rev., 20, p. 377.
Graham, J. D. P. (1951): J. Physiol., 116, p. 36P.
Hazard, R., and Cornec, A. (1952): C.R. Soc. Biol., 146, p. 896.
Lembeck, F., and Strobach, R. (1955): Arch. exp. Path. Pharmak., 228, p. 130.

EVIDENCE IN SUPPORT OF AN AXIAL METABOLIC GRADIENT IN THE EARTHWORM

by B. R. A. O'BRIEN

(From the Department of Histology and Embryology, University of Sydney).

(*Accepted for publication, 11th October, 1956.*)

The concept of metabolic gradients has been used by experimental embryologists for many years. The question of the relationship between these gradients and growth or differentiation remains unanswered. Regeneration is one facet of the problem of growth and differentiation. The class Annelida provides both classical examples of regeneration and excellent material for observation and analysis. Child (1941) and his school have suggested that there exists a definite relationship between the extent of posterior regeneration and an axial metabolic gradient. Much of this work has been criticised by other workers, who either have been unable to obtain consistently similar results, or who claim that the methods used did not warrant the conclusions. The methods used by Child and his school include:

(a) The demonstration of differential susceptibilities to injurious agents such as X-ray, neutral KCN, lithium salts, etc.;

(b) The reduction of oxidised vital dyes by the organism under anaerobic conditions;

(c) Observations on the oxidase catalysis of α-naphthol and dimethyl-para-phenylenediamine;

(d) Variation in potentiometric phenomena;

(e) Estimation of oxygen uptake and carbon dioxide production.

Strelin (1938), using solutions of methylene blue, was unable to demonstrate a reduction gradient in *Limnodrilus* and *Tubifex*. Bröndsted (1937), working with *Planaria*, was unable to obtain a clear gradient picture because of the variable staining of different organs. Bröndsted concluded that differences in the reducing activity of various regions was not related to morphogenetic activity. Hyman (1923), using Winkler's technique, described the existence of a respiratory gradient in planarians, but these results were criticised on the basis that the Winkler method was unsuitable for the problem. In addition, Schearer (1930), using a manometric technique, was unable to verify the presence of an antero-posterior gradient in worms cut across this axis. Parker's work (1929) with *Nereis* and planarian worms led to similar conclusions.

Watanabe and Child (1933) examined the carbon dioxide elimination, indophenol oxidase reaction and differential susceptibility in the worm *Stylochus*, and obtained a definite and consistent U-shaped antero-posterior gradient curve, the anterior and posterior ends exhibiting greater activity than the intermediate zones. Similar results have been obtained by Kawaguti (1934) which agree with the earlier work of Hyman and Galegher (1921), Perkins (1929) and Okada (1929), all of whom describe a U-shaped gradient of respiratory activity. Maloeuf (1936) suggested that differential motor activity of pieces was the cause of the U-shaped respiratory curve and, using very small pieces of earthworm (*Lumbricus*) in order to eliminate this factor, was unable to agree with those mentioned above. More recently, von Bertalanffy (1941) determined the growth gradients in certain planaria and concluded that there was no apparent relationship between metabolic gradients and growth.

In the following work with the earthworms, *Eisenia foetida* and *Octolasium cyaneum*, a definite U-shaped gradient curve was obtained for several metabolic phenomena.

Materials and Methods.

Two species of earthworm were used, *Eisenia foetida* (Sav) and *Octolasium cyaneum* (Sav). *Eisenia foetida* has been maintained in laboratory culture for several years and *Octolasium cyaneum* was collected in the field prior to its use. Tissue preparations (mince or homogenate) were made for the most part from body wall, termed "muscle" and in some experiments, pieces of "whole worm" and preparations of "viscera" were used. The tissues were obtained, cleaned and prepared as previously described (O'Brien, 1948). The body wall, though predominantly muscle, consists of three layers: cuticle, epidermis and a muscular layer. The cuticle is a thin, transparent, non-chitinous, albuminoid layer, made up of two or more thin laminae, the outermost of which is homogenous, the inner layers showing a faint fibrillar structure. The cuticle is considered to be a secretion product of the epidermis. The epidermis is a pseudo-stratified columnar layer, made up of several cell types and resting on a basement membrane. The cellular constituents are: (a) Supporting cells; (b) Basal or replacement cells; (c) Glandular cells of two types, mucous and albuminous; (d) Scattered sensory cells of various types. The muscle layer makes up the greater part of the body wall and consists of two layers, an outer circular and an inner longitudinal. Associated with these main layers are groups of setal muscle fibres, a system of branching and anastomosing connective tissue fibres, and a faintly fibrillated ground substance. Visceral tissue consists of the entire contents of the body cavity, that is the alimentary canal, associated glands, reproductive and excretory structures, and the coelomic fluid with its cellular constituents.

Glycogen was determined by KOH extraction followed by Nelson's estimation for sugar.

Lipoid material is considered to be the fraction obtained by 50/50 alcohol/ether extraction.

Lactic and pyruvic acids were estimated according to the method given in Umbreit, Burris and Stauffer (1946), using a Spekker photoelectric colorimeter over a range of 0 to 30 μg. Anaerobic production of lactic acid, using a glucose substrate, is taken to be a measure of the glycolytic activity of the tissue.

Oxidative activity was estimated using standard Warburg procedure to obtain values for both endogenous oxygen uptake and succinoxidase activity. The reaction systems for both

anaerobic glycolysis and succinoxidase were essentially similar to those for vertebrate muscle. Assay procedures were developed in each case. For the centrifugal fractionation of homogenised tissue, a Servall SSIA centrifuge was used.

RESULTS.

Glycogen distribution.

Glycogen has long been considered the metabolic reserve in the terrestrial annelids (Cuénot (1898), Hertling (1923)). A preliminary analysis showed that "muscle tissue" contained about 4·5 mg./gm. wet weight of tissue and the "viscera" about 7·0 mg/gm.

Fig. 1 represents a series of determinations in each of which twenty large specimens of *Eisenia foetida* were used.

The worms were first cut into the portions indicated. Each portion was then divided into "muscle" and "viscera" and analysed for glycogen. Each portion represents a zone of approximately ten segments. All worms were non-clitellate.

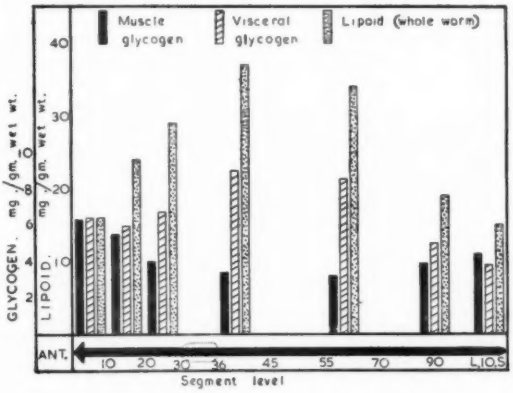

Fig. 1. Muscle glycogen, visceral glycogen and "whole worm" lipoid related to segment level. Clitellar zone extends from segment 29 to 36. Each zone analysed consists of approximately 10 body segments. The posterior zone contains the last 10 segments, usually from 100 to 110. The histograms represent the mean of five separate analyses; for each analysis large non-clitellate specimens of *Eisenia foetida* were used. Glycogen estimations, 30 worms per lot. Lipoid estimations, 20 worms per lot. Results expressed as mg./gm. wet weight of worm tissue.

Alcohol/ether extracted material.

Fat also forms an important metabolic reserve and has been shown to be rapidly used during starvation (Lesser, 1908-10). In the following, that fraction

extracted by 50/50 alcohol/ether is considered to be lipoid. Preliminary analyses showed that "whole worm" contained approximately 3·5 mg. of lipoid per 100 mg. wet weight and that the greater proportion of this is found in the visceral fraction, Table 1.

TABLE 1.

Lipoid material in three different tissue preparations of E. foetida. *The entire worm was used; segment levels not considered.*

Tissue	No. Analyses	No. Worms/Analysis	Mg. lipoid/100 mg. wet wt.
Whole worm	3	10	3·4
'Muscle'	3	20	1·4
'Viscera'	3	20	2·6

The axial distribution of lipoid is shown in Fig. 1. Twenty large, non-clitellate specimens of *E. foetida* were used in each analysis; the muscle and viscera were not separated in this case.

Lactic and pyruvic acid, glycolysis.

Both lactic and pyruvic acid occur free in the tissues, lactic acid in more significant quantities than pyruvic. Table 2 represents analyses of lactic and pyruvic acids in frozen "whole worm" tissue. The worms (*E. foetida*) were frozen alive at a fairly rapid rate, care being taken to adjust the initial freezing, so that no abnormal muscular activity took place. The frozen worms were then cut into portions, as in the experiment above, and analysed.

TABLE 2.

Lactic and pyruvic acid expressed as μ *gm./100 mg. live weight.*

The line ACP represents the worm. A the anterior end, P the posterior end, and C the clitellar zone, including body segments 29 *to* 40. *Each zone contains approximately* 10 *segments. Zone* 5 *includes segments* 60 *to* 70, *zone* 6 *represents the last* 10 *segments.*

A	1	2	3	C	4		5		6	P
L.	18	22	24		17		11·5		9·6	
P.	1·6	1·24	2·9		1·9		1·2		3·3	

Glycolytic activity, as measured by the anaerobic production of lactic acid in the presence of a glucose substrate, was determined in relation to the longitudinal axis of the worm. The endogenous anaerobic lactic production is compared with that produced in the presence of glucose. Thirty large non-clitellate specimens of *E. foetida* were used and the results expressed in Table 3.

TABLE 3.

Muscle homogenate. 200 mg./ml. wet weight. Glycolytic reaction system used with 2 p.c. glucose as substrate. Results expressed as μ gm. lactic/200 mg./60 mins. 30 large non-clitellate worms used. Temp. 27° C. Each zone represents 10 body segments. Zone 5 includes segments 60-70.

System N_2/CO_2	Time A mins.	1	2	3	4	5	6	P
Endogenous	0	23	20	26	18	15	8·5	
Endogenous	60	42	45	38	29	24	17	
Difference		19	25	12	11	9	8·5	
Glucose	60	110	95	80	64	60	85	
Difference		68	50	42	35	36	68	

Endogenous oxygen uptake.

Both minced and homogenised preparations of "muscle" and "viscera" were used. In the homogenised preparations the tissue level was 200 mg. wet weight per flask, instead of the usual 100 mg.

In this series, large clitellate specimens of *E. foetida* were used, and the relatively low activity of the clitellate zone in endogenous preparations, is of interest in that it is relatively high in the succinoxidase assay. Table 4 summarises the results of five experiments in each of which twenty worms were used.

TABLE 4.

Eisenia foetida. *Large clitellate worms. Homogenate contains the equivalent of 200 mg. wet weight of tissue; mince contains 100 mg. wet weight. Zones 1-10 consist of approximately 10 segments, each except 10 which varies somewhat (5-20 in specimens used). Clitellum usually extends from segment 29 to segment 34. 20 worms used in each experiment. Oxygen uptake expressed as μl. 02/100 mg. wet weight/60 mins.*

	A	1	2	3	4 C	5	6	7	8	9	10	P
Homogenate 200 mg. tissue	Muscle	5·3			—	2·6		4·1			7·6	
	Viscera	4·6			2·5	2·8		2·9			3·1	
Mince 100 mg. tissue	Muscle	48	30	27		23		31			44	
	Viscera	12			1	4		5			8	

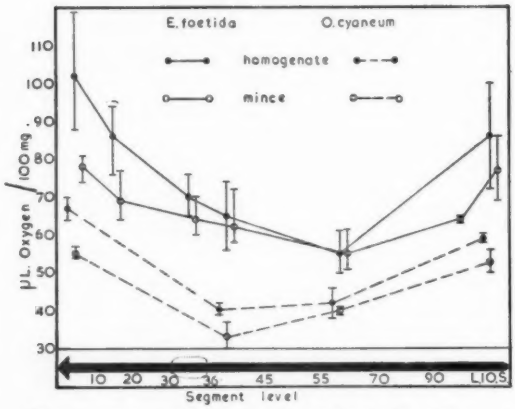

Fig. 2. Succinoxidase activity of the body wall in relation to segment level. Both minced and homogenised tissue obtained at the various levels from *Eisenia foetida* and *Octolasium cyaneum*. Each point on the graph represents the mean of a range expressed by the vertical line through the point. Results expressed as µl. of oxygen absorbed per 100 mg. wet weight of tissue per 60 mins. or its equivalent in the homogenate. Experimental temperature, 27° C. Ten large clitellate worms in each of several experiments.

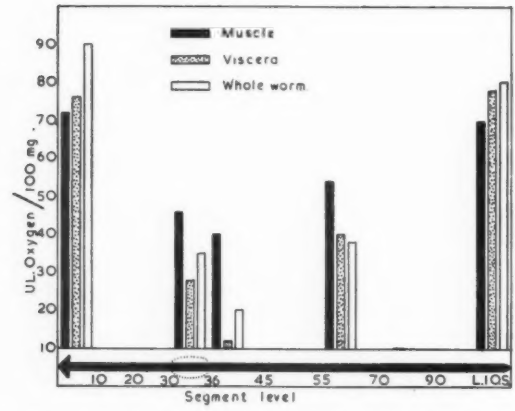

Fig. 3. Succinoxidase activity of three tissue preparations from *Octolasium cyaneum*. Results expressed as µl. of oxygen per 100 mg. wet weight per 60 mins. Experimental temperature, 27° C. 30 large clitellate specimens used.

Succinoxidase activity.

Succinoxidase activity was determined for both minced and homogenised muscle preparations from two species of worm, *Eisenia foetida* and *Octolasium cyaneum*.

These results form Fig. 2. Fig. 3 compares the succinoxidase activity of three different tissue preparations from *O. cyaneum* along the antero-posterior axis.

Centrifugation. Granular fraction.

Preliminary experiments indicated that the muscle succinoxidase activity was largely associated with the 5,000*g*. fraction of the homogenate. Using muscle homogenates from four segment levels along the antero-postero axis, the succinoxidase activity was determined in both species. Ten large worms were used in each case and the assay carried out in micro-Warburg flasks. These results form Table 5.

TABLE 5.

10 large non-clitellate worms used in each case. 5,000g. fraction. Activity expressed as $\mu l.O_2$ *absorbed/100 mg. wet weight/60 mins. Temperature 27° C.*

Species	A	1	2	3	4	C	5	6	7	8	9	10	P
E. foetida		74			43				31			58	
O. cyaneum		59			38				35			46	

DISCUSSION.

Hertling (1923) described the occurrence of glycogen, in the parietal peritoneum generally, along the vessel mesenteries, and in association with the ventral nerve cord. Stephenson also states that it is present in the body wall musculature and in the walls of the alimentary canal and dorsal vessel. Finally, glycogen granules have been described in the chloragogen cells where they occur among the small granule fraction, rather than in association with the chloragosomes (Cuénot, 1898, and Hertling, 1923). The analyses of Fig. 1 indicate that the glycogen content of the body wall is slightly higher in the anterior region and fairly uniform from the immediate pre-clitellar region to the posterior end. The visceral glycogen follows an inverted U-shaped curve, in which significantly higher values occur in the post-clitellar region. In this region histological examination shows that it is here the chlorogogue tissue reaches its greatest development.

There appears a distinct antero-posterior gradient in lipoid content. Histological structure would suggest a distribution curve of this type. In zones 1 and 2, the alimentary canal consists of a thick muscular pharynx and oesophagus, a crop and a gizzard. The epithelium of these structures is probably non-absorptive; that of the pharynx and oesophagus is made up of low, poorly granular, columnar cells which show no evidence of lipoid granules. The crop is a large, thin-walled diverticulum in which food does not appear to undergo any significant change (Stephenson, 1950), certainly not fat absorption. The gizzard is a thick-walled, highly muscular organ, lined with a thick chitinous layer, whose main function is one of trituration of food material. The coelomic cavity around this section of the canal contains coelomic fluid, eleocytes, nephridial and glandular tissue, none of which, with the exception of eleocytes, contain obvious lipoid granules.

In view of this structure, one would expect that the eleocytes are the only source of extra muscular lipoid, in contrast to the intestinal zones where the lipoid absorptive epithelium contributes the greatest proportion of the total fat of the worm. Towards the posterior end, intestinal sacculations are absent and the epithelium appears to develop a definite cuticular layer: both situations could conceivably result in less absorption and consequently less lipoid. Thus in both anterior and posterior regions the greater part of the lipoid material is probably muscle tissue phospholipid (several analyses of 'muscle' show an average value of 39 μg. of phospholipid per 100 mg. wet weight) whereas in the middle section, this is considerably augmented by the intracellular lipoid of the intestinal epithelium and coelomic eleocytes.

The distribution of free lactic acid declines antero-posteriorly from region 2, the anterior region 1 containing slightly less than regions 2 and 3. Thus the analyses of three types of substances, all related to energy metabolism, show similar types of distribution curve, either one showing a steady antero-posterior decline such as muscle glycogen, or one in which the curve rises to a maximum followed by a decline, such as visceral lipoid and lactic acid. However, if metabolic activity rather than metabolic substrate or product is measured, a distinctly different gradient curve is obtained. In this case, both in anaerobic and aerobic metabolism, a distinct U-shaped gradient results. Table 3 demonstrates this for the anaerobic metabolism of glucose, and in Figs. 2 and 3 a similar gradient in succinoxidase activity is clearly shown.

These results suggest that, in the investigation of a gradient in activity or in function, an estimation of endogenous substrates or products might not be sufficient, and that the reaction system, however crude, should be investigated also. Since relative values are the important ones, the inefficiency of the system set up may not vitiate the results.

Both species demonstrate the same type of gradient in oxidase activity. It is of interest that the preparations from *E. foetida* were considerably more

active than those from *O. cyaneum*. An examination of the body wall tissue with a microspectroscope showed that cytochrome C absorption bands could be distinguished in *E. foetida* but not in *O. cyaneum*, although the latter certainly had some endogenous cytochromes or equivalent carrier, as could be deduced from substrate and cytochrome C addition experiments. However, at both substrate and cytochrome C saturation, *E. foetida* muscle was more active than *O. cyaneum* (unpublished experiments).

The arguments against the gradient phenomena, which postulate the differential muscular activity of small pieces of "whole" worm as being the sole causative agent, would seem wrong in the light of these results, as tissue homogenate would certainly preclude this argument. In no experiment was Maloeuf's result duplicated, although in the case of minced tissue, conditions somewhat similar to his "very small pieces" would be attained.

Finally, the succinoxidase assay of the fraction obtained by centrifugation suggests that there is some fundamental activity gradient along the antero-posterior axis, and this, expressed here as succinoxidase activity, appears in whole cell preparations, acellular homogenates, and in the particulate fraction obtained by centrifugation.

SUMMARY.

The antero-posterior distribution of glycogen, lipoid, lactic and pyruvic acids, has been determined for the earthworm—*Eisenia foetida* (Sav).

A U-shaped, axial, gradient curve has been demonstrated for anaerobic production of lactic acid, using glucose as a substrate in homogenised body wall tissue.

Succinoxidase activity in minced and homogenised tissues shows a similar U-shaped gradient along the antero-posterior axis in two species of earthworms — *E. foetida* and *Octolasium cyaneum* (Sav).

The antero-posterior gradient in succinoxidase activity of the particulate fraction of homogenised muscle, obtained by centrifugation at 5,000*g*., is also a U-shaped curve.

The experimental results support the claim, denied by some, that a U-shaped antero-posterior metabolic gradient is present in the earthworm.

Acknowledgments. This work has been aided by a grant from the University of Sydney Cancer Research Fund.

REFERENCES.

Bertalanffy, L. v. (1941): Biol. Gener. (Vienna), 15, p. 295.
Brachet, J. (1950): "Chemical Embryology," Interscience, New York.
Bröndsted, H. V. (1937): Quoted from Brachet (1950).
Child, C. M. (1941): "Patterns and problems of development," Univ. of Chicago Press, Chicago.
Cuénot, L. (1898): Arch. Biol., 15, p. 79.

Hertling, H. (1923): Z. wiss. Zool., 120, p. 147.
Hyman, L. (1923): J. exp. Zool., 37, p. 47.
Hyman, L., and Galegher, A. E. (1921): *Ibid.*, 34, p. 1.
Kawaguti, C. (1932): Mem. Fac. Sci. Taihoku., Imp. Univ., 7, p. 57.
Maloeuf, N. S. R. (1936): Biol. Z., 56, p. 429.
O'Brien, B. R. A. (1948): Proc. Linn. Soc. (N.S.W.), 72, p. 367.
Okada, Y. K. (1929): Roux. Archiv. Entw. Mech. Organ., 115, p. 542.
Parker, G. H. (1929): Brit. Jour. exp. Biol., 6, p. 412.
Perkins, M. (1929): Nature, 124, p. 616.
Schearer, C. (1930): J. exp. Biol., 7, p. 260.
Stephenson, J. (1950): "The Oligochaeta," Clarendon Press, Oxford.
Strelin, G. S. (1938): Arch. russes Anat. Histol. Embryol., 19, p. 226. (Biol. Abstracts 1939, 13, 11467.)
Umbreit, W. W., Burris, R. H., and Stauffer, J. F. (1946): "Manometric techniques and related methods for the study of tissue metabolism," Burgess Publ., Minneapolis.
Watanabe, Y., and Child, C. M. (1933): Physiol. Zool., 6, p. 452.